AD_____

Award Number: DAMD17-02-1-0022

TITLE: Elucidation of a Novel Cell Death Mechanism in Prostate Epithelial Cells

PRINCIPAL INVESTIGATOR: Linda G. Baum, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of California
Los Angeles, California 90024-1406

REPORT DATE: December 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2004	Final (19 Nov 2001 - 18 Nov 2004)

4. TITLE AND SUBTITLE
Elucidation of a Novel Cell Death Mechanism in Prostate Epithelial Cells

5. FUNDING NUMBERS
DAMD17-02-1-0022

6. AUTHOR(S)
Linda G. Baum, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California
Los Angeles, California 90024-1406

E-Mail: lbaum@mednet.ucla.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Tumor cell resistance to apoptosis and immune attack are obstacles to effective prostate cancer therapy. Androgen dependent LNCaP prostate cancer cells are sensitive to apoptosis induced by galectin-1, a human lectin that is abundant in prostate stroma. In contrast, androgen independent LNCaP, DU145 and PC-3 cells are resistant to galectin-1 induced death and express galectin-1 on the cell surface. Galectin-1 binds to specific saccharide ligands on LNCaP cells to trigger cell death; susceptibility to galectin-1 requires O-linked glycans on glycoproteins, while N-glycans are not required for galectin-1 induced cell death. Galecti-1 resistance in androgen independent LNCaP cells correlates with decreased expression of a specific glycosyltransferase, C2GnT, that creates O-glycan ligands recognized by galectin-1. Blocking Oglycan elongation by expressing a competing glycosyltransferase, ST3Gal I, renders LNCaP cells resistant to galectin-1 death. Galectin-1 resistant DU145 and PC-3 cells can kill adherent T cells via cell surface gal-1. Moreover, PC-3 cells secrete a factor that up-regulates gal-1 expression by endothelial cells, which then can kill adherent T cells. Thus, enhancing galectin-1 prostate cancer cell death may allow novel therapeutic approaches to manipulate tumor cell glycosylation to overcome tumor cell resistance to apoptosis and to prevent tumor evasion of the immune response.

14. SUBJECT TERMS
Apoptosis, cell death, galectin-1, glycosylation, sialyltransferase

15. NUMBER OF PAGES
30

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Promoting tumor cell death in different anatomic sites and tissues is an important goal in controlling the spread of cancer. We are examining apoptosis, or programmed cell death, induced by a human lectin termed galectin-1. Galectins are a family of mammalian lectins, or carbohydrate binding proteins, with multiple functions, including positive and negative regulation of cell death. Galectin-1 is abundant in many organs, especially in prostate stroma. While galectin-1 was initially reported to kill subsets of B and T lymphocytes,, additional reports have demonstrated that an androgen-dependent prostate cancer cell line undergoes apoptosis after binding soluble galectin-1, while two androgen-independent prostate cancer cell lines are resistant to galectin-1 induced death. As resistance to apoptosis is a hallmark of many types of cancers, we have investigated the mechanisms governing susceptibility or resistance of various prostate cancer cell lines to galectin-1 induced apoptosis. Regulating prostate cancer cell death will allow the development of novel therapeutic approaches to eliminate tumor cells.

Body

The approved Specific Aims and Statement of Work for this project are below. In our three years of funding, we have made substantial progress in all the Tasks listed, as described following the Specific Aims and Statement of Work.

Specific Aims: 1) Characterize features of galectin-1 death in prostate cancer cell lines. 2) Identify the expression pattern of glycosyltransferase genes that regulate galectin-1 cell death in prostate cancer cells, and determine if regulated glycosyltransferase expression controls cell death. 3) Examine whether, as observed in T cells, anti-apoptotic galectin-3 inhibits galectin-1 induced death of prostate cells, and determine if the two galectins compete for common receptors. 4) Characterize the cell surface receptors for gal-1 on prostate cancer cells and compare the pattern of expression of these receptors on galectin-1 sensitive and gal-1 resistant prostate cancer cell lines. Determine the requirement for specific cell surface receptors in galectin-1 mediated death of prostate cells. 5) Investigate the intracellular galectin-1 death pathway, and identify associations between prostate-specific cell surface receptors and common intracellular death pathway components.

Statement of Work

Task 1. To characterize features of galectin-1 death in prostate cancer cell lines (months 1-3).
a. Examine hallmarks of cell death, including membrane lipid assymetry, cell permeability, loss of mitochondrial membrane potential and DNA fragmentation. (months 1-3)
b. Examine calcium flux, caspase activation and cytochrome C release, to identify novel features of the galectin-1 death pathway in prostate cancer cells. (months 1-3)

Task 2. To identify the roles of specific glycosyltransferase genes that regulate galectin-1 cell death in prostate cancer cells (months 1-18).
a. Examine expression patterns of ST6Gal I, ST3Gal I, FucT VII, GnTV and C2GnT glycosyltransferases in galectin-1 sensitive and insensitive LnCaP cell lines (months 1-6).
b. Determine effects of expressing ST3Gal I and FucT VII that mask and C2GnT that create galectin-1 saccharide ligands on prostate cell susceptibility to galectin-1 (months 6-18).
c. Examine prostate cancer biopsies for the pattern of glycosyltransferase expression in primary prostate cancers (months 6-12).

Task 3. To determine whether galectin-3 opposes the effects of galectin-1 in prostate cancer cell lines (months 6-18).
a. Express galectin-3 in a galectin-1 sensitive LnCaP cell line (months 6-9).
b. Determine effect of galectin-3 overexpression on galectin-1 susceptibility (months 9-12).
c. Perform competition experiments with soluble recombinant galectin-1 and -3 (months 12-18).

Task 4. To examine the requirement for specific prostate cell surface glycoprotein receptors for galectin-1 induced death (months 12-36).
a. Following isolation and characterization of receptors, determine receptor expression pattern on galectin-1 resistant cells (months 12-24).
b. Characterize receptor domains that are required for sending the death signal
c. Examine requirements for specific receptors by antibody inhibition, anti-sense transfection or expression of dominant negative receptor constructs in galectin-1 susceptible cells or by expression in galectin-1 resistant cells, (months 24-36).

Task 5. To investigate the intracellular galectin-1 death pathway (months 12-36).
a. Determine effects of protein kinase C and protein phosphatase inhibitors on galectin-1 susceptibility (months 12-18).
b. Identify additional candidate signaling molecules based on identities of cell surface receptors determined in Task 4 (months 18-30).
c. Determine requirement for specific signaling molecules by expression of relevant wildtype or mutant constructs in specific cell lines (months 18-36).
d. Identify association between cell surface receptors and signaling molecules by immunoprecipitation and confocal immunofluorescent microscopy (months 24-36).

Aim 1. Characterize features of galectin-1 death in prostate cancer cell lines

In the first two years of funding we completed **Task 1**. In **Task 1a**, we confirmed that LNCaP cells treated with galectin-1 demonstrate many hallmarks of cell death, including DNA fragmentation, uptake of propidium iodide, and plasma membrane lipid asymmetry as demonstrated by annexin V binding, as shown in Fig. 1. In contrast, DU145 cells (Fig. 1) and PC-3 cells (data not shown) are resistant to galectin-1 death.

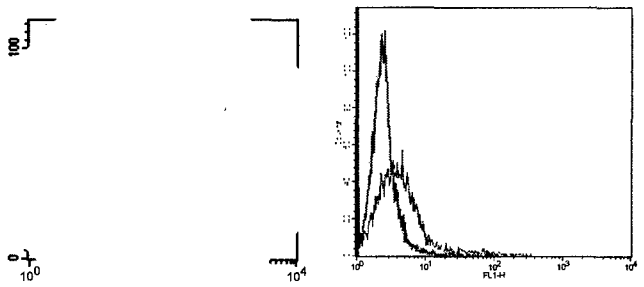


Figure 1. Galectin-1 kills androgen dependent LNCaP cells, but not androgen-independent DU145 cells. Adherent LNCaP cells (left) or DU145 cells (right) were treated with media (red), buffer control (yellow) or 20 μM galectin-1 (blue) for 6 hours. Cells were detached from the plate with gentle pipetting and apoptosis measured by TUNEL labeling to detect fragmented DNA (x-axis). The y-axis represents cell number.

In **Task 1b**, our proposal initially focused on features of caspase-mediated cell death. However, we have recently published that in T cells, galectin-1 induced apoptosis is caspase independent and does not involve early release of mitochondrial cytochrome c (1). Thus, we did not examine a caspase/cytochrome c dependent death pathway in LNCaP cells treated with galectin-1. Rather, we applied a novel observation we have made demonstrating that the serine/threonine phosphatase PP2A is involved in galectin-1 induced T cell death, as PP2A

inhibitors block cell death (Hernandez and Baum, unpublished observations). This will be described in detail in Aim 5, below.

Aim 2. Identify the expression pattern of glycosyltransferase genes that regulate galectin-1 cell death and determine if regulated glycosyltransferase expression controls cell death.

In year 1, we completed **Task 2a**, examining expression patterns of a variety of glycosyltransferase enzymes that could create or modify oligosaccharide ligands for galectin-1. We established a collaboration with Dr. Pirko Vihko at the University of Oulu, Finland, to use microarray analysis to examine patterns of glycosyltransferase expression in androgen dependent and androgen independent prostate cancer cell lines. In year 1, we determined that androgen-dependent LNCaP cells that die to galectin-1 express the core 2 GnT glycosyltransferase that is essential for galectin-1 induced death of T cells, while an androgen-independent subclone of LNCaP that is resistant to galectin-1 induced death expressed very low levels of C2GnT mRNA.

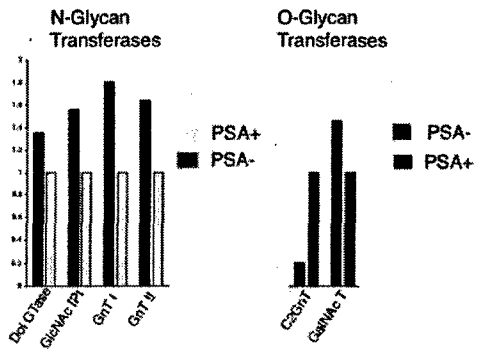


Figure 2. Comparison of glycosyltransferase expression between androgen dependent (PSA+) and androgen independent (PSA-) LNCaP cells. (left panel) Androgen independent LNCaP cells (red) had modestly higher levels of a variety of N-glycan modifying enzymes compared to androgen dependent LNCaP cells (yellow). (right panel) In contrast, androgen independent LNCaP cells had markedly reduced expression of C2GnT enzyme, that elongates O-glycans on cell surface glycoproteins.

This was a striking and important finding, as the C2GnT enzyme is important for regulating galectin-1 induced death of T cells. Our finding of common glycosyltransferase regulation of galectin-1 mediated cell death in T cells and in prostate cancer cells was remarkable, given that prostate cells express none of the polypeptides that bear core 2 O-glycans on T cells. Based on the microarray data, we focused on modifying O-glycans in **Task 2b**.

In years 2- 3, we completed **Task 2b**, identifying glycosyltransferases that would regulate prostate cell susceptibility to galectin-1. We first directly demonstrated that O-glycans are involved in signaling galectin-1 death. As a screen for glycan function, we used inhibitors of specific glycosylation pathways. Inhibiting N-glycan elongation and processing with the inhibitor deoxymannojirimycin had no effect on LNCaP susceptibility to galectin-1 (data not shown). In contrast, removing terminal sialic acid residues that can mask O-glycans by treating cells with benzyl-alpha-GalNAc increased LNCaP cell susceptibility to galectin-1 induced death by almost 2-fold; death of cells reached almost 100% (Table I).

We then wished to enzymatically regulate O-glycan accessibility and creation of core 2 O-glycans. We used two approaches, 1) down-regulating C2GnT by siRNA expression and 2) overexpressing ST3Gal I enzyme that competes with the C2GnT, to block elongation of core 2 O-glycans. The latter approach, overexpressing the ST3Gal I, has been more successful. ST3Gal I expression in LNCaP cells resulted in blocking extension of O-glycans by capping core 1 O-glycans with sialic acid, as shown by loss of PNA staining. Importantly, as shown in Table I, expression of the ST3Gal I significantly reduced the susceptibility of LNCaP cells to galectin-1. In combination, the benzyl-alpha-GalNAc and ST3Gal I overexpression data demonstrate that O-glycans are critical ligands in the galectin-1 death pathway in these cells, and that masking or

loss of these O-glycan ligands may contribute to apoptosis resistance and tumor progression in prostate cancer.

Table I – O-glycans are required for galectin-1 induced cell death

Expose O-glycans with benzyl-alpha-GalNAc		Mask O-glycans with ST3Gal I	
Treatment	% cell death	Treatment	% cell death
Buffer	5%	Buffer	2%
Galectin-1/vector	69%	Galectin-1/vector	55%
Galectin-1/B-a-GalNAc	95%	Galectin-1/ST3Gal I	14%

This work was presented at a Gordon Conference on Glycobiology in March, 2003. Subsequently, Fukuda and co-workers have published an abstract demonstrating that C2GnT expression in prostate cancer biopsies is an independent predictor of cancer progression (2). This address a goal of **Task 2c**, determining if the pattern of glycosyltransferase expression that protects against galectin-1 induced cell death in vitro is also seen in primary prostate cancers.

Aim 3. Examine whether, as observed in T cells, anti-apoptotic galectin-3 inhibits galectin-1 induced death of prostate cells, and determine if the two galectins compete for common receptors.

Table II Galectin expression in prostate cancer cell lines

Cell line	Galectin-1	Galectin-3
LNCaP	No	No
PC-3	Yes	Yes
DU145	Yes	Yes
LAPC-4	No	Yes

Galectin-1 and galectin-3 expression were examined in human prostate cancer cell lines by Western blotting. The LAPC-4 human prostate cancer xenograft cell line was obtained from Dr. Charles Sawyers, Dept. of Medicine, UCLA School of Medicine.

As a first step in Aim 3, we screened a panel of prostate cancer cell lines for expression of galectin-1 and galectin-3 and susceptibility to galectin-1 induced cell death. As shown in Table II, this includes cell lines generated in the lab of Dr. Charles Sawyers at UCLA, with whom we collaborate. Notably, the only galectin-3 negative cell line is LNCaP, which is also the only cell line that we have identified to date that is susceptible to galectin-1 induced cell death. This observation supports our hypothesis that, as we have found in T cells, galectin-3 expression in prostate cancer may protect cell from apoptotic death; this role for galectin-3 has now been established in breast cancer (3).

In collaboration with Fu-Tong Liu (UC Davis) and Mike Teitell (UCLA), we have also demonstrated a role for galectin-3 in conferring resistance to apoptosis in diffuse large B cell lymphoma (4). Importantly, we also found that the C-terminal domain of gal-3 acted as a dominant negative (DN) and abolished galectin-3 mediated apoptosis resistance, consistent with recent work by John and co-workers that the C-terminal domain of galectin-3 inhibited tumor growth and metastasis in breast cancer in a mouse model (5). We are currently overexpressing galectin-3 in LNCaP cells as previously described (6) to determine if this blocks galectin-1 mediated cell death; this completes **Task 3a** and we will soon complete **Task 3b**. We are also expressing DN galectin-3 C-terminal domain in these cells and in other cells in Table 1 that express endogenous galectin-3, and determine if this enhances susceptibility of these cells to apoptosis. This may provide a novel approach for overcoming prostate cancer resistance to apoptosis in patients.

For **Task 3c**, we are continuing to ask if extracellular galectin-1 and galectin-3 would compete for the same glycoprotein counterreceptors on the cell surface. For these assays, we have produced biotinylated galectin-1 and galectin-3 and developed a binding assay in which one unlabeled galectin is used to compete the binding of the labeled galectin. While our DAMD funding for this current project is completed, we will continue to work on **Task 3** to complete this important set of experiments.

Aim 4. Characterize the cell surface receptors for galectin-1 on prostate cancer cells

Our initial approach in **Task 4a** was to focus on candidate glycoprotein receptors expressed on prostate cancer cells. As galectin-1 has been shown to bind to the cancer mucin CA-125, and recent data from our lab suggests that some galectins may recognize CD44, we proposed the prostate cancer mucin Muc18 and CD44 as potential receptors involved in galectin-1. However, the galectin-1 susceptible LNCaP cells express neither of these potential receptors.

Thus, as an alternative approach, we focused on another prostate cell glycoprotein, CD10, that is expressed on galectin-1 sensitive LNCaP cells but not on galectin-1 resistant DU145 or PC-3 cells. Importantly, CD10 associates with the intracellular actin scaffolding ezrin-radixin-moesin (ERM) proteins (7). On T cells, CD43 is an important receptor involved in regulating susceptibility to galectin-1 death (Hernandez and Baum, manuscript in preparation). CD43 is a major binding partner of ERM proteins in T cells, suggesting that binding to glycoproteins that connect to the ERM scaffold is critical for triggering cell death. To determine if CD10 on LNCaP cells is important for triggering galectin-1 induced cell death, in Task **4c**, we are currently using an siRNA approach to down-regulate CD10 expression, to determine if this confers resistance to galectin-1. If decreased CD10 expression confers resistance to galectin-1, we will continue with Task 4b by determining if the cytosolic portion of CD10 that associates with ERM proteins is required for galectin-1 to signal cell death. In addition, we have prepared a galectin-1 affinity column and are in the process of isolating LNCaP membrane glycoproteins that bind to galectin-1, as we have done previously (8), to continue to identify galectin-1 receptors that are potentially important for regulating susceptibility to cell death. As described for Aim 3, while the current project funding has ended, we are continuing with this exciting and important project.

Importantly, in year 2, we moved into an exciting and productive new direction in examining the functions of tumor cell galectin-1 (see Appendix 1). Work of Castronovo and co-workers had demonstrated abundant deposition of galectin-1 in the stroma of prostate cancers, with increasing galectin-1 deposition positively correlating with disease progression and poor

patient outcome (9, 10); this group had proposed that galectin-1 acts as an "immunologic shield" around tumors. We directly addressed this hypothesis, by growing galectin-1 producing stromal cells on plastic or on the extracellular matrix material Matrigel. We found several surprising results. First, stromal cells secreted increased amounts of galectin-1 when growing on Matrigel, indicating that matrix material increased secretion of galectin-1 by mass action removal from the stromal cells. Second, when Lec8 stromal cells that secrete misfolded galectin-1 were grown on Matrigel, galectin-1 folded properly on the Matrigel, as demonstrated by binding and functional assays. Third, galectin-1 secreted onto the matrix could kill T cells without stromal contact. Fourth, 10-fold less galectin-1 was required to kill T cells when the galectin-1 was presented by extracellular matrix, compared to soluble galectin-1. Fifth, death of T cells occurred very rapidly, with cells becoming annexin V^+ within 60 minutes of binding to galectin-1 on matrix; this implies that tumors that secrete galectin-1 will thwart any immunotherapy approaches to controlling cancer. This work, published in 2004 (11, Appendix 1), has profound implications for our understanding of tumor defenses against the immune system.

In year 3, we have extended these studies to examine the effects of prostate cancer cells in regulating galectin-1 expression and evasion of the immune response. We have found that T cells adherent to galectin-1 expressing DU145 cells undergo apoptosis. This demonstrates that increased expression of galectin-1 in prostate cancer can blunt the immune response by 1) directly killing T cells that bind the cancer cells (Fig. 3), or 2) killing T cells in the matrix surrounding the tumor (11, Appendix 1).

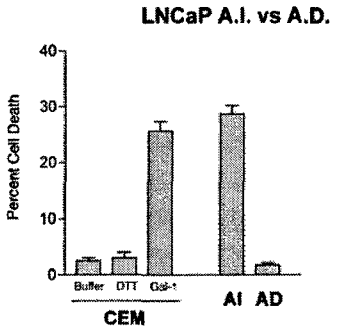


Figure 3. Prostate cells expressing galectin-1 kill susceptible T cells. (left side of panel as a control, CEM T cells were treated with buffer, media control containing DTT or soluble recombinant galectin-1 and cell death determined by TUNEL staining to detect fragmented DNA. (right side of panel) DU145 cells express cell surface galectin-1. Androgen dependent (AD) or androgen independent (AI) LNCaP cells were bound to the DU145 cells for 1 hr. Cells were detached and cell death determined by TUNEL labeling to detect fragmented DNA.

We next examined effects of galectin-1 expression by endothelial cells exposed to prostate cancer cells. Clause et al found that conditioned medium (CM) from androgen independent DU145 or PC-3 prostate cancer cells induced galectin-1 expression by endothelial cells (10). We confirmed this observation and found that CM-treated endothelial cells, expressing high levels of galectin-1, killed adherent T cells in a glycan- dependent manner.

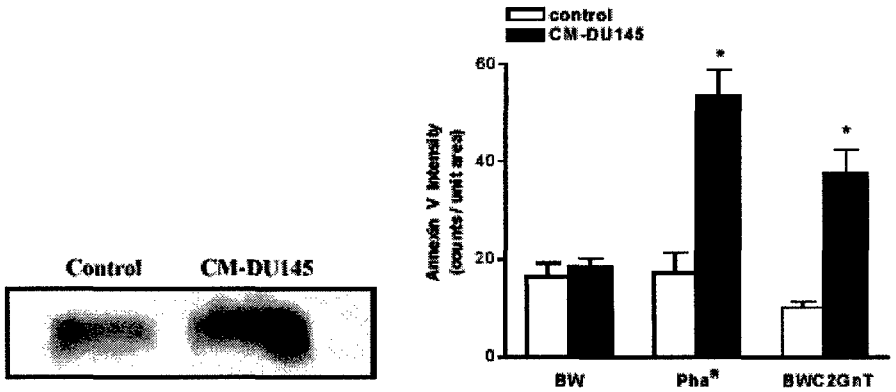


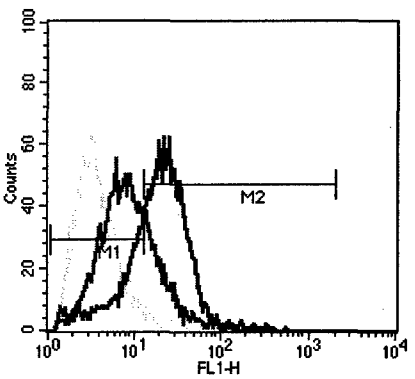
Figure 4. Human endothelial cells treated with DU145 conditioned medium (CM) increase gal-1 expression and kill adherent T cells. (left panel) Western blot of cell extracts of human endothelial cells treated with medium alone (Control) or CM, probed with polyclonal anti-gal-1 antiserum. Equal cell number equivalents were loaded in each lane. (right panel) BW5147 (BW) T cells do not express C2GnT and are resistant to galectin-1 death, while PhaR cells express C2GnT and are susceptible to galectin-1. BW cells transfected with C2GnT express core 2 O-glycans and are also susceptible to galectin-1. All three cell lines demonstrated equal levels of adhesion to control or CM-treated endothelial cells (data not shown). In contrast, the two T cell lines expressing C2GnT underwent apoptosis, as detected by annexin V binding, when bound to CM-treated endothelial cells that expressed high levels of galectin-1.

These data demonstrate a third manner by which prostate cancer cells can up-regulate galectin-1 expression to kill infiltrating T cells. In addition to T cell killing by galectin-1 on the surface of prostate cancer cells and secreted into extracellular matrix, induction of galectin-1 expression on endothelial cells adjacent to prostate cancer cells may kill T cells before they can leave the blood and infiltrate a tumor. This work is currently being completed and prepared for publication.

Aim 5. Investigate the intracellular galectin-1 death pathway, and identify associations between prostate-specific cell surface receptors and common intracellular death pathway components

As described on page 6, we found that PP2A inhibits T cell death induced by galectin-1. To identify intracellular components of the galectin-1 death pathway in prostate cancer cells in **Task 5a**, we examined the effects of PP2A inhibitors on death of LNCaP cells by galectin-1.

Figure 5. PP2A is important for galectin-1 induced death of LNCaP cells. Cells were treated with (red) or without (blue) calyculin A prior to treatment with galectin-1, or with buffer control (yellow) for 5 hrs. Cell death was determined by TUNEL labeling detected by flow cytometry. Calyculin A treatment reduced the fraction of apoptotic cells from 73% for control-treated cells to 23% for calyculin A-treated cells.



To examine the role of PP2A in galectin-1 induced LNCaP cell death, we treated cells with or without calyculin A, a PP2A inhibitor, prior to the addition of galectin-1. As shown in Fig. 5, cells treated with calyculin A demonstrated a dramatic reduction in cell death compared to cells treated with vehicle alone. The finding that PP2A appears to be required for galectin-1 induced cell death in both prostate cancer cells and T cells raises the exciting possibility that, like the common requirement for C2GnT expression and O-glycan elongation to confer galectin-1 susceptibility in prostate cancer cells and T cells, the intracellular galectin-1 death pathway may also be common between the two cell types. In addition, work by Thompson and co-workers has shown that PP2A inhibition via caveolin signaling was important for survival of LNCaP cells (12), additionally supporting a role for PP2A activity in cell death.

Key Research Accomplishments

- Demonstration that androgen-dependent prostate cell lines do not express galectin-1, while androgen-independent prostate cell lines synthesize galectin-1 and can export it to the cell surface.
- Establishment of apoptosis assays that demonstrate susceptibility of androgen dependent LNCaP cells and resistance of androgen independent PC-3 and DU145 cells lines to galectin-1 induced cell death, measuring membrane lipid asymmetry, plasma membrane permeability and nuclear DNA fragmentation.
- Identification of a class of cell surface oligosaccharides, the O-linked glycans, which control prostate cancer cell susceptibility to galectin-1 induced death, using pharmacologic inhibitors of glycoprotein processing.
- Establishment of a collaboration with Dr. Pirrko Vihko, Univeristy of Oulu, Finland, to examine differential gene expression that relates to galectin-1 susceptibility between androgen-dependent and androgen-independent LNCaP cells.
- Characterization of altered glycosyltransferase expression, specifically reduced expression of the C2GnT enzyme, in androgen-independent, galectin-1 resistant cells, compared to androgen-dependent, galectin-1 susceptible cells.
- Identification of a single glycosyltransferase, the C2GnT, that appears to control susceptibility to galectin-1 death in both lymphoid and prostate cancer cells, indicating a common mechanism to regulate of galectin-1 susceptibility in diverse tissue types.
- Expression of the ST3Gal I in LNCaP cells, and demonstration that the expressed enzyme is functional by reduced PNA binding detected by flow cytometry.
- Demonstration that masking O-glycans by expression of ST3Gal I reduced LNCaP susceptibility to galectin-1 induced death.
- Establishment of a collaboration with Dr. Charles Sawyers, UCLA, to examine differential gene expression that relates to galectin-1 susceptibility in other androgen-dependent and androgen-independent pairs of prostate cancer cell lines, to extend the work we have done on LNCaP cells
- Characterization of different prostate cancer cell lines for expression of various galectin family members.
- Exclusion of Muc18 and CD44 as candidate receptors essential for galectin-1 cell death, and identification of PNA-binding cell surface glycoproteins as potential receptors essential for galectin-1 cell death.
- Demonstration that cell usrface galectin-1 on DU145, PC-3 and androgen independent LNCaP cells can kill adherent T cells.
- Demonstration that galectin-1 presented on extracellular matrix can kill T cells, and that galectin-1 presented on matrix kills T cells 10-fold more efficiently than we have observed with soluble galectin-1.
- Characterization of increased galectin-1 expression by endothelial cells induced by conditioned medium from PC-3 and DU145 prostate cancer cells.
- Determination that increased galectin-1 expression by endothelial cells treated with conditioned medium results in increased death of adherent T cells, regulated by T cell expression of core 2 O-glycans.
- Identification of protein phosphatase 2A as an important intracellular signaling molecule involved in galectin-1 induced cell death.

Reportable outcomes

Presentation of this work in two posters, by Dr. James He and Dr. Hector Valenzuela, at the 2003 6th Annual San Diego Glycobiology Symposium.

Presentation of this work by the P.I. in an invited platform presentation at the 2003 Gordon Conference on Glycobiology.

Presentation of parts of this work in a poster by a graduate student, Joseph Hernandez, at the 2003 Gordon Conference on Glycobiology.

Presentation of this work by the P.I. as the keynote speaker at the 23rd Biennial Cornea Conference at the Massachusetts Eye and Ear Infirmary, Boston, MA, 2003.

Presentation of this work by the P.I. as an invited speaker at the NanoSystems Symposium, California NanoSystems Institute, UCLA, Los Angeles, CA.

Presentation of this work by the P.I as an invited speaker at the 2004 7th Annual San Diego Glycobiology Symposium.

Presentation of this work by the P.I. as an invited speaker at Ramon Areces International Symposium on Receptors of the Immune System, Barcelona, Spain, 2004

Publication of a manuscript describing matrix-associated galectin-1 induction of T cell death.
He J and Baum LG (2004) Presentation of galectin-1 by extracellular matrix triggers T cell death. *Journal of Biological Chemistry* 279:4705-4712

Publication of a review article on the effects of galectin-1 binding to cell surface glyconjugates.
Brewer CF, Miceli MC and Baum LG (2002) Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide mediated cellular interactions. *Current Opinion in Structural Biology* 12:616-623.

Two manuscripts are currently in preparation.
Valenzuela H, Porvari K, Vihko P, and Baum LG. Glycosylation regulates susceptibility of prostate cancer cells to galectin-1.
Will be submitted in early 2005

He J and Baum LG. Prostate cancer cells increase galectin-1 expression by endothelial cells to regulate T cell death and migration.
Will be submitted in early 2005.

Submission of a renewal application in February 2004, that received a ranking of 22%ile; we will submit a revision of this application in February 2005 to continue our work in this exciting field.

Conclusions

In this three-year project, we have made substantial and rapid progress in defining the features and mechanisms of galectin-1 induced death of prostate cancer cells. Importantly, we have proven one of our major hypotheses, i.e. that glycosylation controls prostate cell susceptibility to apoptosis, as we have found that over-expression of the ST3Gal I glycosyltransferase that masks core 1 O-glycans, significantly reduced the susceptibility of LNCaP cells to galectin-1 induced death. Moreover, this work demonstrates that common mechanisms such as glycosylation of cell surface glycoproteins regulate susceptibility to galectin-1 death in different cell types, T cells and prostate cancer cells.

Another important accomplishment is our demonstration that galectin-1 in extracellular matrix kills T cells, thus validating the hypothesis that galectin-1 secreted into prostate cancer can act as an "immunologic shield" protecting the tumor from immune attack. Furthermore, our recent finding that prostate cancer cells increase galectin-1 expression by tumor associated endothelial cells to facilitate apoptosis of adherent T cells demonstrates yet another mechanism by which prostate cancer cells can use galectin-1 to evade an immune attack.

A renewal application for this project submitted February 2004 received a 22%ile score and was not funded; however, we have responded to the reviewers' comments and will be submitting a revised application for February 2005.

"So what section": Our work demonstrates that controlling glycosylation may provide a novel therapeutic approach for treating prostate cancer. Altering glycosylation may make prostate cancer cells susceptible to endogenous galectin-1 and to pharmacologic galectin-1, facilitating elimination of tumor cells *in vivo* through apoptosis. As the galectin-1 death pathway appears to be distinct from that mediated by other apoptotic agents, this will allow a synergistic therapeutic approach to eliniating prostate cancer cells. Moreover, our work shows that tumors develop diverse molecular mechanisms to protect themselves from cell death. In addition to escaping cell death by altering cellular glycosylation, prostate cancer cells that can resist galectin-1 apoptosis can secrete galectin-1 to kill invading immune cells attempting to attack the tumor. By controlling the mechanisms that regulate prostate cancer cell susceptibility to death, we can exploit two potential pathways to control prostate cancer, i.e. direct killing of the cancer cells by apoptosis inducing agents such as galectin-1, and enhanced immune attack of the tumor by lymphocytes.

References

1. Hahn HP, Pang M, He J, Hernandez JD, Yang R-Y, Li LY, Wang X, Liu F-T, Baum LG (2004) Nuclear translocation of Endonuclease G in caspase- and cytochrome c-independent galectin-1 induced T cell death. Cell Death and Differentiation 11:1277-86
2. Ohyama C, Hagisawa S, Hatakeyama S, Arai Y, Fukuda M (2003) Core 2 β1,6-N-acetylglucosaminyltransferase (C2GnT) expression in human prostate cancer: a predictor for non-organ confined disease and biochemical relapse after radical prostatectomy. Glycobiol 13: abstract 143
3. Moon BK, Lee YJ, Battle P, Jessup JM, Raz A, Kim HR (2001) Galectin-3 protects human breast carcinoma cells against nitric oxide-induced apoptosis: implication of galectin-3 function during metastasis. Am J Pathol 159:1055-60
4. Hoyer KK, Pang M, Kuwabara I, Liu F-T, Said JW, Baum LG, Teitell MA (2004) An anti-apoptotic role for galectin-3 in diffuse large B cell lymphomas. Am J Pathol 164:893-902
5. John CM, Leffler H, Kahl-Knutsson B, Svensson I, Jarvis GA (2003) Truncated galectin-3 inhibits tumor growth and metastasis in orthotopic nude mouse model of human breast cancer. Clin Cancer Res 9:2374-83
6. Ellerhorst JA, Stephens LC, Nguyen T, Xu XC (2002) Effects of galectin-3 expression on growth and tumorigenicity of the prostate cancer cell line LNCaP. Prostate 50:64-70
7. Iwase A, Shen R, Navarro D, Nanus DM (2004) Direct binding of neutral endopeptidase 24.11 to ezrin/radixin/moesin (ERM) proteins competes with the interaction of CD44 with ERM proteins. J Biol Chem 279:11898-905
8. Pace KE, Lee C, Stewart PL and Baum LG (1999) Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. J Immunol 163:3801-11
9. van den Brule FA, Waltregny D, Castronovo V (2001) Increased expression of galectin-1 in carcinoma-associated stroma predicts poor outcome in prostate carcinoma patients. J Pathol 193:80-7
10. Clausse N, van den Brule F, Waltregny D, Garnier F, Castronovo V (1999) Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell-cell adhesion. Angiogenesis 3:317-25
11. He J and Baum LG (2004) Presentation of galectin-1 by extracellular matrix triggers T cell death. J Biol Chem 279:4705-4712
12. Li L, Ren CH, Tahir SA, Ren C, Thompson TC (2003) Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol. 23:9389-404

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 6, Issue of February 6, pp. 4705–4712, 2004
Printed in U.S.A.

Presentation of Galectin-1 by Extracellular Matrix Triggers T Cell Death*

Received for publication, October 10, 2003, and in revised form, November 13, 2003
Published, JBC Papers in Press, November 14, 2003, DOI 10.1074/jbc.M311183200

Jiale He and Linda G. Baum‡§

From the Department of Pathology and ‡Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, California 90095

Apoptotic elimination of T cells at sites of inflammation or infiltration into tumors limits an effective immune response. T cell apoptosis can be initiated by a variety of triggers, including galectin-1, a soluble, secreted lectin that binds to oligosaccharide ligands on cell surface glycoproteins, or to oligosaccharide ligands on extracellular matrix glycoproteins in tissue stroma. Although galectin-1 has no transmembrane domain and is secreted from cells that make it, it is not clear if galectin-1 functions as a soluble death trigger *in vivo*. We examined the ability of stromal cells secreting galectin-1 to kill T cells. Although the stromal cells synthesized abundant galectin-1, the majority of the galectin-1 remained bound to the cell surface, and stromal cell-associated galectin-1 killed bound T cells. In contrast, insufficient amounts of functional galectin-1 were released from the stromal cells into the media to kill T cells in the absence of contact with stromal cells. However, when stromal cells were grown on Matrigel, a mixture of extracellular matrix proteins, or on permeable membranes above Matrigel, secreted galectin-1 bound to Matrigel and killed T cells without stromal cell contact. Ten-fold less galectin-1 on Matrigel was sufficient to kill adherent T cells compared with soluble galectin-1. These results demonstrate that galectin-1 in extracellular matrix is able to directly kill susceptible T cells. Because increased galectin-1 deposition in tumor stroma occurs with tumor progression in various types of cancer, galectin-1 in stroma may act locally in the apoptotic elimination of infiltrating T cells during an immune response.

Various factors regulate lymphocyte survival. This regulation may have positive effects, *e.g.* prevention of self-recognition and autoimmune disease, or negative effects, *e.g.* cancer cells can kill infiltrating lymphocytes that would attack the tumor (1–3). Galectins are a family of mammalian lectins with a variety of immunoregulatory functions, including control of lymphocyte death (4–9). Galectin-1, the first member of the family to be described, has a broad repertoire of immunoregulatory effects. Galectin-1 regulates the inflammatory responses of neutrophils, mast cells, and macrophages and also associates with components of the complement system (10, 11). Galectin-1 induces apoptosis of macrophages, thymocytes, T cells, and B cells (9). Pircher and co-workers (12) detected an increase in galectin-1 synthesis after activation of murine T cells and suggested that galectin-1 can act as an autocrine negative regulatory "cytokine," killing T cells to terminate an immune response. Galectin-1 is highly expressed in $CD4^+/CD25^+$ regulatory T cells that suppress immune responses compared with conventional CD4 cells (13). *In vivo*, galectin-1 therapy ameliorated disease in models of hepatitis, nephritis, arthritis, inflammatory bowel disease, and multiple sclerosis (9, 14).

Galectin-1 is expressed in a variety of cell types, including thymic epithelial cells, endothelial cells, dendritic cells, macrophages, fibroblasts, and bone marrow stromal cells (4–6, 8, 9, 15, 16). There is increased galectin-1 expression in many types of cancer, including colon, breast, ovary and prostate carcinomas, and aggressive glioblastomas (8) and increased accumulation of galectin-1 in stroma surrounding tumor cells in ovarian and prostate carcinoma (17, 18). Numerous ligands for galectin-1 have been described in different tissues; in extracellular matrix, galectin-1 binds to laminin, fibronectin, and vitronectin and is proposed to facilitate tumor cell invasion and migration through stroma (4, 5, 8, 17–19). Although galectin-1 may function in tumorigenesis and metastasis, galectin-1 expression by tumors may also modulate the immune response to the tumor (4).

The mechanisms by which galectin-1 mediates these immunomodulatory effects have not been elucidated. In particular, it is not known whether galectin-1 can diffuse away from the cell that secretes it to act as a soluble "cytokine" or whether galectin-1 requires direct cell-cell contact to exert its effects. In the former case, secretion of large amounts of soluble galectin-1 by tumors could have a global immunosuppressive effect. In the latter case, whereas galectin-1 synthesis may be increased in a tumor, the immunosuppressive effects would be limited to the vicinity of the tumor. Moreover, although several studies have documented accumulation of galectin-1 in extracellular matrix, the ability of extracellular matrix-associated galectin-1 to effect T cell death is not known.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Recombinant human galectin-1 and polyclonal rabbit antiserum to human galectin-1 were prepared as described (15). The following reagents were purchased as indicated: annexin V/propidium iodide (PI)[1] and galectin-3 (R&D Systems, Minneapolis, MN), dithiothreitol (Fisher), 10× PBS and bovine serum albumin (Sigma), Ficoll-Paque (Amersham Biosciences), horseradish peroxidase-chromogen kit (Biomedia, Foster, CA), Matrigel (BD Biosciences), mouse anti-rabbit-horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA), Texas Red-goat anti-rabbit IgG, FITC-annexin V (Molecular Probes, Eugene, OR), and sulfo-NHS-biotin (Pierce).

* This work was supported by National Institutes of Health Grant GM63281, Department of Defense Grant 17-02-1-0022, and by the Cancer Research Institute (to L. G. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 310-206-5985; Fax: 310-206-0657; E-mail: lbaum@mednet.ucla.edu.

[1] The abbreviations used are: PI, propidium iodide; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

Cells and Culture Conditions—The BW5147Pha^{R}2.1 cell line (Pha^{R}2.1), gift of Dr. M. Pierce, and the θ35 thymic stromal epithelial cell line, a gift of Dr. K. Dorshkind, were cultured as described (20). Chinese hamster ovary (CHO) cells and Lec8-CHO (American Type Tissue Collection, Manassas, VA) were grown in α-minimal essential medium (Invitrogen) with 10% fetal bovine serum. For transwell experiments, 4.5×10^5 θ35 cells, CHO, or Lec8 cells in 3 ml of media were plated in the bottom chamber of 6-well plates (Costar, Corning, NY) for 24 h. To assess T cell death, viability, and proliferation, fresh medium was added to the stromal cells, and 2.25×10^5 Pha^{R}2.1 cells in 1.5 ml of media were placed in the upper inserts (0.4 μm pore size, BD Biosciences); cell counts and trypan blue exclusion assays were performed at the indicated times. Pha^{R}2.1 cell death was assessed by flow cytometry using annexin V and PI as described (20).

To deposit galectin-1 on Matrigel, 2×10^5 stromal cells in 1.5 ml of media were plated directly on solidified Matrigel or placed in the upper inserts, and 3 ml of medium was added to the lower wells over 100 μl of solidified Matrigel on glass coverslips (Fisher). 100 μl of liquid Matrigel was pipetted onto coverslips in each well at 4 °C. Plates were brought to room temperature to solidify the gel, and the gel was air-dried for 1 h. 100 μl of media was added to the solidified gel to keep the gel hydrated. Galectin-1 bound to Matrigel was detected by immunohistochemistry and quantitated by ELISA (below).

To bind recombinant galectin-1 or galectin-3 directly to Matrigel, 100 μl of galectin at the indicated concentrations in PBS, 0.1% bovine serum albumin was added to the surface of the solidified Matrigel for 1 h. The Matrigel was washed once with PBS before T cell binding assays. No unbound galectin-1 or galectin-3 was detected in the wash buffer after Matrigel binding, indicating that all added galectin bound to the Matrigel.

Cell Conjugate Assays and Confocal Microscopy—To assess T cell death by cell-cell contact, 10^5 Pha^{R}2.1 cells were incubated for 1 h with subconfluent (~50%) monolayers (~2×10^5 cells) of θ35 cells, CHO, and Lec8 cells plated on coverslips in 6-well plates. To assess T cell death by T cell matrix contact, 10^5 Pha^{R}2.1 cells were added to Matrigel solidified on coverslips and allowed to bind the Matrigel for 1 h. Unbound cells were removed by washing once with PBS. 100 μl of annexin V-FITC/PI in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$) was added to the wells for 20 min at 20 °C. Coverslips were washed with PBS, fixed with 2% paraformaldehyde for 30 min at 4 °C, washed with PBS, quenched with 0.2 M glycine in PBS for 10 min at 4 °C, and mounted onto slides with 25 μl of Prolong anti-fade mounting medium (Molecular Probes).

For immunohistochemistry cell conjugates or cell-Matrigel layers were prepared on coverslips as above, blocked with 10% goat serum, and incubated with either anti-galectin-1 antiserum or with normal rabbit serum, all diluted 1:100 in PBS with 2% goat serum. After washing, bound antibody was detected with Texas Red-goat anti-rabbit IgG for 1 h at 20 °C for fluorescence microscopy or with mouse anti-rabbit horseradish peroxidase (1:200) and 3-amino-9-ethylcarbazole for light microscopy. After washing, coverslips were mounted to slides as above.

For fluorescence microscopy samples were excited at 488 and 568 nm with argon and krypton lasers for FITC and Texas Red or PI, respectively, and the light emitted between 525 and 540 nm was recorded for FITC and above 630 nm for Texas Red and PI. Slides were visualized on a Fluoview laser scanning confocal microscope (Olympus America Inc., Melville, NY) using the 100× objective. Dual emission fluorescent images were collected in separate channels. Images were processed using the Fluoview image analysis software (version 2.1.39). To compensate the microscope single stains with corresponding negative controls were performed. Quantification of Pha^{R}2.1 cell death was evaluated in 50–100 conjugates randomly selected in 5–8 microscopic fields for each experiment. The percent annexin V-positive T cells was calculated as the number of annexin V-positive T cells over the total number of adherent T cells.

ELISA Assay for Galectin-1—Anti-galectin-1 IgG was purified from rabbit polyclonal anti-galectin-1 antiserum on a Proteus Protein A spin column (Pro-Chem, Acton, MA). Purified anti-galectin-1 IgG was diluted in PBS to 10 μg/ml, and 100 μl/well was added to 96-well enhanced protein binding ELISA plates (Immulon 2, Thermo Electron Corp., Franklin, MA) overnight at 4 °C. Wells were washed 3 times with wash buffer (1× PBS, 0.05% Tween 20) and blocked with blocking buffer (10% fetal bovine serum in PBS) for 1 h at room temperature. After blocking, recombinant galectin-1, conditioned media, or cell lysates were added for 1 h at room temperature. Wells were washed 3 times, biotinylated anti-galectin-1 IgG (10 μg/ml in blocking buffer) prepared as in Amano *et al.* (21) was added for 1 h, and wells were washed 3 times. Streptavidin-horseradish peroxidase (1:500 in blocking buffer) was added for 30 min at room temperature. After washing, 200 μl of 0.4 mg/ml *o*-phenylenediamine dihydrochloride was added to each well, and the absorbance of each well was read at 490 nm using a microplate reader (Bio-Rad Model 550). Absorbance values were converted to protein concentrations based on a galectin-1 standard curve. To quantify galectin-1 bound to Matrigel, biotinylated anti-galectin-1 IgG was added directly to the Matrigel and allowed to bind for 1 h. Color was developed as above, and 200-μl aliquots were transferred to 96-well ELISA plates.

Statistical Analysis—Data were analyzed by student's *t* test, one-way analysis of variance analysis. Significance was considered at $p < 0.05$, and data are presented as the mean ± S.E.

RESULTS

Direct T cell binding to cells that express galectin-1, such as human and murine thymic stromal cells and activated endothelial cells, can trigger T cell death (15, 20). However, it has been suggested that galectin-1 can act as a cytokine or soluble factor *in vivo* to trigger T cell death and inhibit T cell proliferation (12, 22). To directly examine whether cells that synthesize galectin-1 can kill T cells in the absence of cell-cell contact, we examined the ability of the murine θ35 thymic stromal cells to kill murine Pha^{R}2.1 T cells (Fig. 1*A*). We observed that >50% of Pha^{R}2.1 T cells in contact with θ35 cells became annexin V-positive within 1 h of binding to θ35 cells. The annexin V-positive cells also demonstrated membrane blebbing and clustering of annexin V on apoptotic blebs, as we have described previously (20). In addition to annexin V staining, cells were also labeled with propidium iodide to detect the later stage of cell death characterized by loss of membrane integrity. 45% of the annexin V-positive cells were also labeled with propidium iodide (data not shown), demonstrating that several hallmarks of cell death were present after the T cells bound to the thymic stromal cells.

Pha^{R}2.1 cell death appeared to require direct contact with θ35 cells, as T cells on the plastic tissue culture surface adjacent to, but not in contact with the θ35 cells showed no cell death above background (Figs. 1, *A* and *B*). This suggested that the θ35 cells did not secrete sufficient gal-1 to kill nearby T cells that were not in direct contact with the θ35 stromal cells. In addition, T cell death was galectin-1-dependent, as death was virtually abolished by the addition of anti-galectin-1 antiserum (Fig. 1*A*).

To examine the requirement for contact between galectin-1-expressing cells and T cells for T cell death, we plated θ35 thymic stromal cells in the bottom wells of transwell plates and added Pha^{R}2.1 T cells to the top wells. This would allow any galectin-1 secreted by the θ35 cells to diffuse through the semipermeable membrane separating the upper and lower wells but would not allow T cell-stromal cell contact. In contrast to experiments in which direct T cell-stromal cell contact triggered T cell death within 1 h (Fig. 1), we detected no loss of T cell viability when T cells were grown in the upper wells of transwell plates above θ35 cells for 24, 48, or 72 h; the viability of T cells cultured over θ35 cells was essentially identical to the viability of T cells cultured over bottom wells containing media alone (Table I). Similar results were seen at 1, 3, 6, 12, and 144 h of culture (data not shown).

We then examined CHO and Lec8 cells as galectin-1-secreting cells. CHO cells make abundant galectin-1 that is secreted from the cell and binds back to oligosaccharide ligands on the cell surface (23). Lec8 cells are a CHO mutant lacking UDP-galactose transporter activity (24); as no galactose residues are added to cell surface glycoproteins or glycolipids, there are no available oligosaccharide ligands for galectin-1 on the surface of Lec8 cells, and all the galectin-1 is secreted into the media (23). θ35, CHO, and Lec8 cells all make abundant galectin-1, between 40 and 60 μg/mg of total cellular protein; we detected

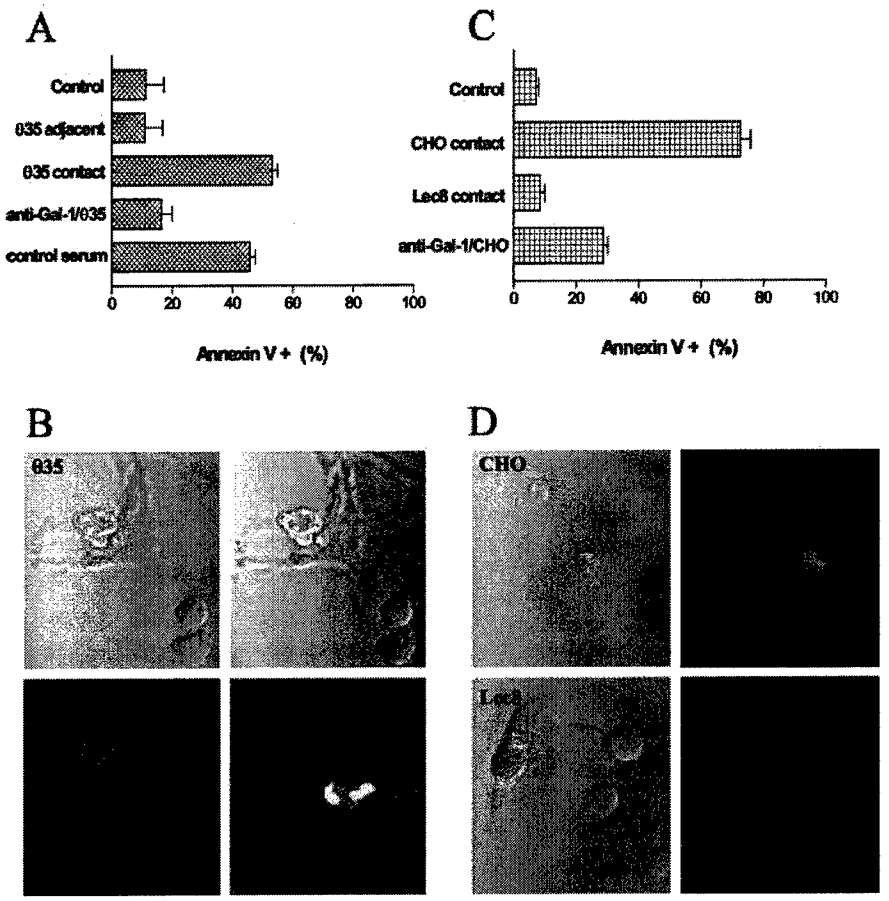


FIG. 1. **Galectin-1 on the stromal cell surface kills adherent T cells.** *A* and *B*, $Pha^R2.1$ T cells were added to subconfluent monolayers of θ35 thymic stromal cells for 1 h. Cell death was assessed by annexin V-FITC binding. The % annexin V^+ T cells in contact with or adjacent to θ35 cells was determined for 50–100 T cells in 5–8 microscopic fields. The *top bar* in *A* indicates % annexin V^+ T cells in the absence of θ35 cells. Death was inhibited by the addition of anti-galectin-1 antiserum (*anti-Gal-1*). *Panel B* is a phase image of an adherent θ35 cell and a T cell (*upper left*), annexin V-FITC staining of the T cell (*lower left*), overlay of the phase and fluorescent images (*upper right*), and galectin-1 expression by a θ35 cell (*red*) and annexin V-FITC binding by an adherent T cell (*green*) (*lower right*). *C* and *D*, $Pha^R2.1$ T cell death was assessed as above on CHO or Lec8 cells. The *top bar* in *C* indicates the percent annexin V^+ T cells in the absence of CHO or Lec8 cells. *Panel D* shows phase images of T cells bound to CHO and Lec8 cells; numerous annexin V^+ T cells were bound to CHO cells, whereas T cells bound to Lec8 cells did not become annexin V^+. In *A* and *C* values are the mean of triplicate samples in a representative experiment.

TABLE I
Galectin-1 secretion and effect on T cell viability

	24 h		48 h		72 h	
	Viab[a]	Gal-1[b]	Viab	Gal-1	Viab	Gal-1
Media	100		100		100	
θ35	97.9	7.3	98.5	1.9	97.0	0.9
CHO	100.5	48.8	100.7	53.6	102.7	47.4
Lec8	100.6	40.5	99.1	79.5	99.7	106.8

[a] % $Pha^R2.1$ viability compared to control.
[b] Galectin-1 concentration (μg/ml) in medium.

galectin-1 on the surface of θ35 and CHO but not Lec 8 cells (data not shown).

>70% of $Pha^R2.1$ T cells that adhered to CHO cells became annexin V-positive within 1 h of contact with CHO cells. In contrast, we detected no annexin V binding to T cells in contact with Lec8 cells that had no detectable cell surface galectin-1 (Figs. 1, *C* and *D*). As we had observed with the θ35 cells, in addition to annexin V binding, we observed membrane blebbing of T cells in contact with CHO cells (Fig. 1*D*) but no membrane blebbing of T cells in contact with Lec8 cells. When the T cells were added to the CHO cells, 61% of the annexin V-positive cells also labeled with propidium iodide, indicating loss of membrane integrity (data not shown).

Again, as we observed with θ35 cells, there was no loss of viability of $Pha^R2.1$ T cells in the upper wells of the transwell plates when either CHO or Lec8 cells were plated in the bottom wells compared with bottom wells containing media alone (Table I). We measured the amount of galectin-1 secreted from θ35, CHO, and Lec8 cells into the media in the bottom of the transwell plates (Table I). The θ35 cells secreted barely detectable amounts of galectin-1 into the media, whereas both the CHO and Lec8 cells secreted appreciable quantities of galectin-1 into the media (47 and 107 μg/ml, respectively). Indeed, galectin-1 secretion by Lec8 cells was increased compared with CHO cells, because the Lec8 cells did not bind back the secreted galectin-1. However, although these cells made abundant galectin-1, the amount of soluble galectin-1 secreted by either CHO or Lec8 cells into the media was not sufficient to reach the concentration required for $Pha^R2.1$ T cell death. We have determined that concentrations above 150 μg/ml (10 μM) soluble galectin-1 are optimal for triggering T cell death, most likely because the K_d of the galectin-1 dimer is in this range, and dimeric galectin-1 is required to induce death (15, 19). Thus, the requirement for stromal cell-T cell contact demonstrated in Fig. 1 may reflect an ability of the stromal cells to concentrate the galectin-1 at the cell-cell interface, rather than an active process by stromal cells in triggering T cell death.

To confirm that soluble galectin-1 could kill $Pha^R2.1$ cells in the transwell system, we added increasing concentrations of soluble galectin-1 to the lower wells of the transwell plates and measured the death of T cells in the upper wells. As shown in

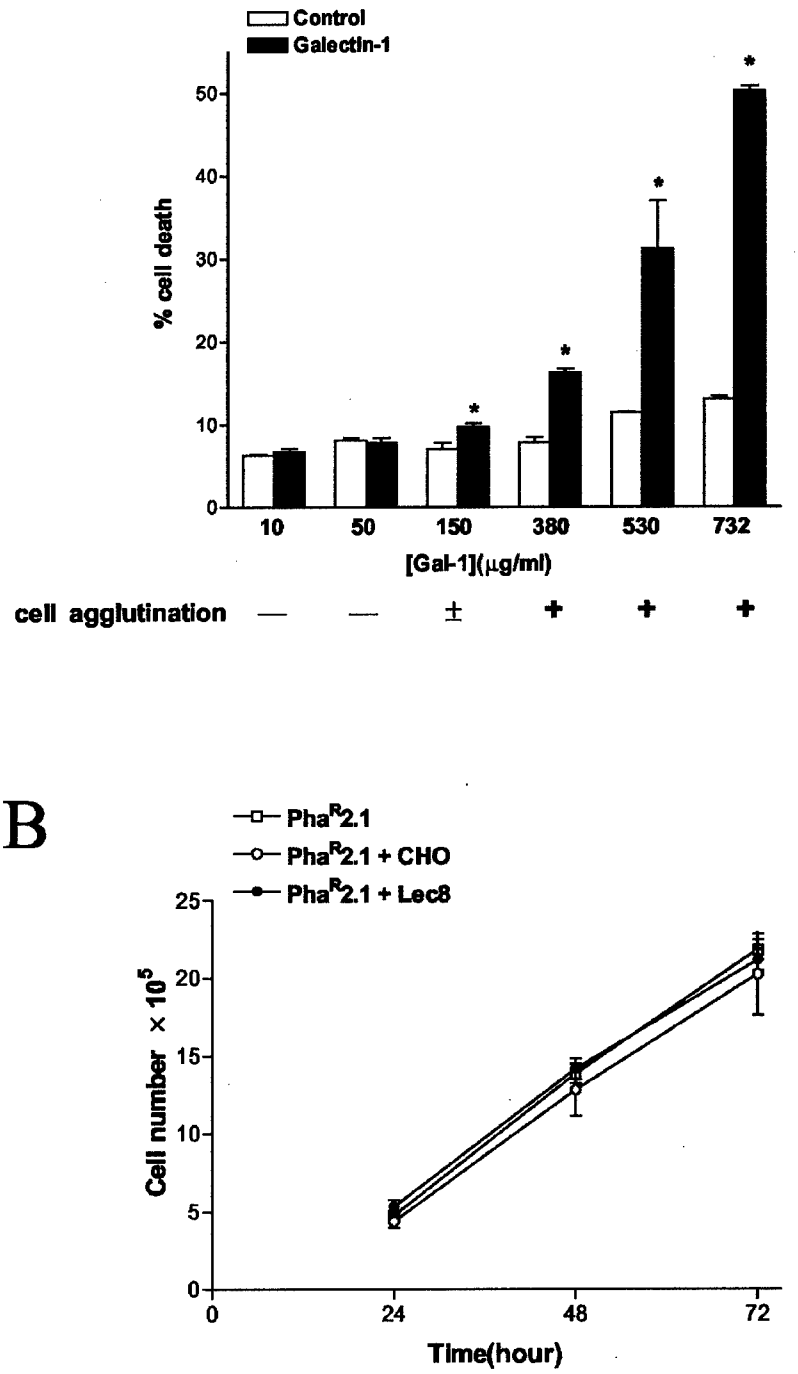


FIG. 2. **T cell death and proliferation.** *A*, to ensure that soluble galectin-1 can cross the transwell membrane to kill T cells, increasing concentrations of recombinant galectin-1 were added to the bottom wells, with Pha^{R}2.1 T cells in the upper wells. After 24 h, the galectin-1 concentration in the media in the upper and lower wells was determined; values for the upper and lower wells were essentially equivalent, and the mean is shown. Agglutination was observed by light microscopy, and T cell death was assessed by flow cytometry using annexin V and PI. *B*, secreted galectin-1 had no effect on Pha^{R}2.1 T cell proliferation. Pha^{R}2.1 cells were cultured in the upper well inserts, with CHO, Lec8, or no cells in the bottom wells. Pha^{R}2.1 cell number was determined at indicated times. Results in *A* and *B* are the mean of triplicate samples.

Fig. 2*A*, no death of Pha^{R}2.1 T cells in the upper wells of the transwell chambers was observed until the concentration of soluble galectin-1 in the wells was in excess of 150 μg/ml, which was also the concentration at which T cell agglutination began to occur, confirming multimeric binding of galectin-1. In these experiments appreciable T cell death was seen at galectin-1 concentrations of 380 μg/ml and above. Thus, very high concentrations of galectin-1 would have to be released from stromal cells in the bottom wells to kill T cells only 0.9 mm across the transwell plate.

In addition, there was no positive or negative effect on T cell proliferation over 72 h by galectin-1 secreted from stromal cells. The numbers of Pha^{R}2.1 cells in the upper wells of the transwell plates were essentially identical at 24, 48, and 72 h whether the lower wells contained CHO cells, Lec8 cells, or no stromal cells (Fig. 2*B*). Galectin-1 stimulates neural and hepatic cell proliferation at concentrations as low as 5 pg/ml (25, 26); although CHO and Lec8 cells secreted 40–100 μg/ml of galectin-1 (Table I), we detected no effect on T cell proliferation.

Thus, the ability of stromal cells to kill T cells via galectin-1

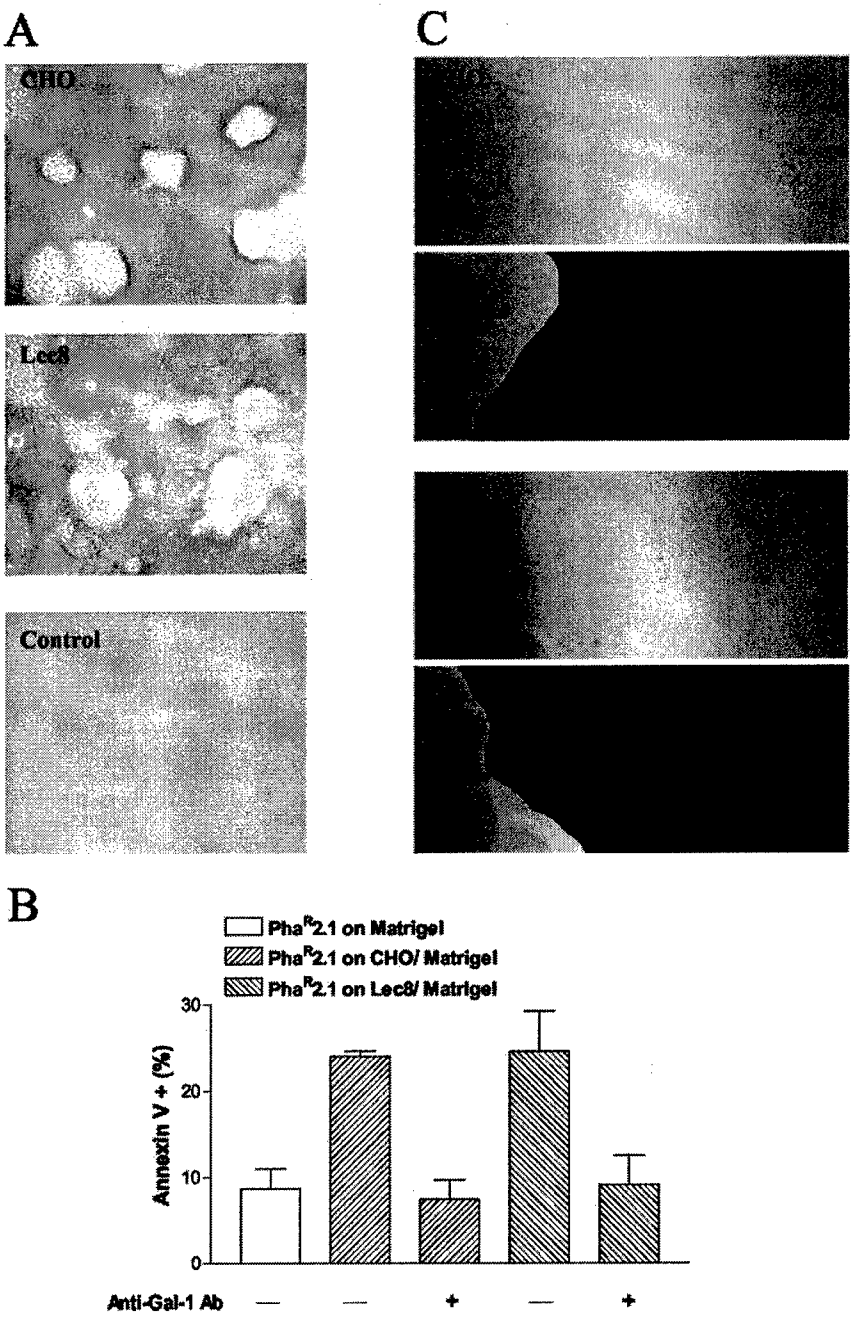


FIG. 3. **Stromal cells secrete galectin-1 that binds to Matrigel and kills T cells.** *A*, CHO or Lec8 cells were plated on solidified Matrigel for 72 h, and galectin-1 was detected by immunohistochemistry (*red*); no galectin-1 was detected in the absence of stromal cells (*Control*). *B*, Pha^{R}2.1 cells were added to Matrigel, on which CHO or Lec8 cells had grown for 72 h. After 1 h, % annexin V$^+$ T cells was determined; only T cells bound to Matrigel but not in direct contact with CHO or Lec8 cells (shown in *C*) were counted. Death was inhibited by anti-gal-1. Values are the mean of triplicate determinations. *C*, annexin V binding to T cells on Matrigel adjacent to CHO or Lec8 cells. In each set, the *top panel* is a phase micrograph of a cluster of CHO or Lec8 cells (*left*) with individual T cells (*right*); the *bottom panel* is an overlay of phase and fluorescent images to delineate the edge of the CHO or Lec8 cluster and demonstrates FITC-annexin V binding to T cells.

may relate to the ability of the stromal cell surface to concentrate and present galectin-1. Because several extracellular matrix glycoproteins, such as laminin, fibronectin, and vitronectin bind galectin-1, cells secreting galectin-1 could also deposit the lectin on the surrounding extracellular matrix; increased galectin-1 accumulation in tumor stroma could kill infiltrating T cells (17, 18). To examine galectin-1 secretion into extracellular matrix, we used Matrigel, which contains laminin, collagen IV, entactin, nidogen, and heparan sulfate proteoglycans. By immunoblot analysis we did not detect any endogenous galectin-1 in Matrigel (data not shown).

CHO and Lec8 cells were plated on Matrigel, and the deposition of galectin-1 on the Matrigel was detected immunohistochemically. Abundant galectin-1 was deposited on Matrigel cultured with either CHO or Lec8 cells (Fig. 3*A*). We added T cells to plates containing Matrigel and either CHO or Lec8 cells. Matrigel coated by galectin-1 produced by CHO cells killed a significant fraction of bound Pha^{R}2.1 T cells (Fig. 3*B*). Importantly, T cells analyzed in Fig. 3*B* were adjacent to, but did not appear to be in contact with the adherent CHO or Lec8 cells (Fig. 3*C*). Thus, galectin-1 in extracellular matrix as well as on the cell surface could trigger T cell death, although the level of T cell death on the matrix was less than that observed for direct T cell-CHO cell contact (Fig. 1, *A* and *C*).

Surprisingly, we also observed T cell death when T cells were added to Matrigel coated by galectin-1 from Lec8 cells (Fig. 3*B*), although no death above background was observed when T cells bound directly to Lec8 cells (Fig. 1*C*). Growth of Lec8 cells directly on Matrigel apparently allowed the secreted galectin-1 to bind to glycoconjugate ligands in the extracellular matrix and retain carbohydrate binding activity (Fig. 3*A*). On Matrigel coated with galectin-1 from either CHO or Lec8 cells, galectin-1 was responsible for T cell death, since there was no death of T cells plated on Matrigel alone, and anti-galectin-1 antiserum reduced T cell death to background levels.

To exclude the possibility that death of T cells on galectin-

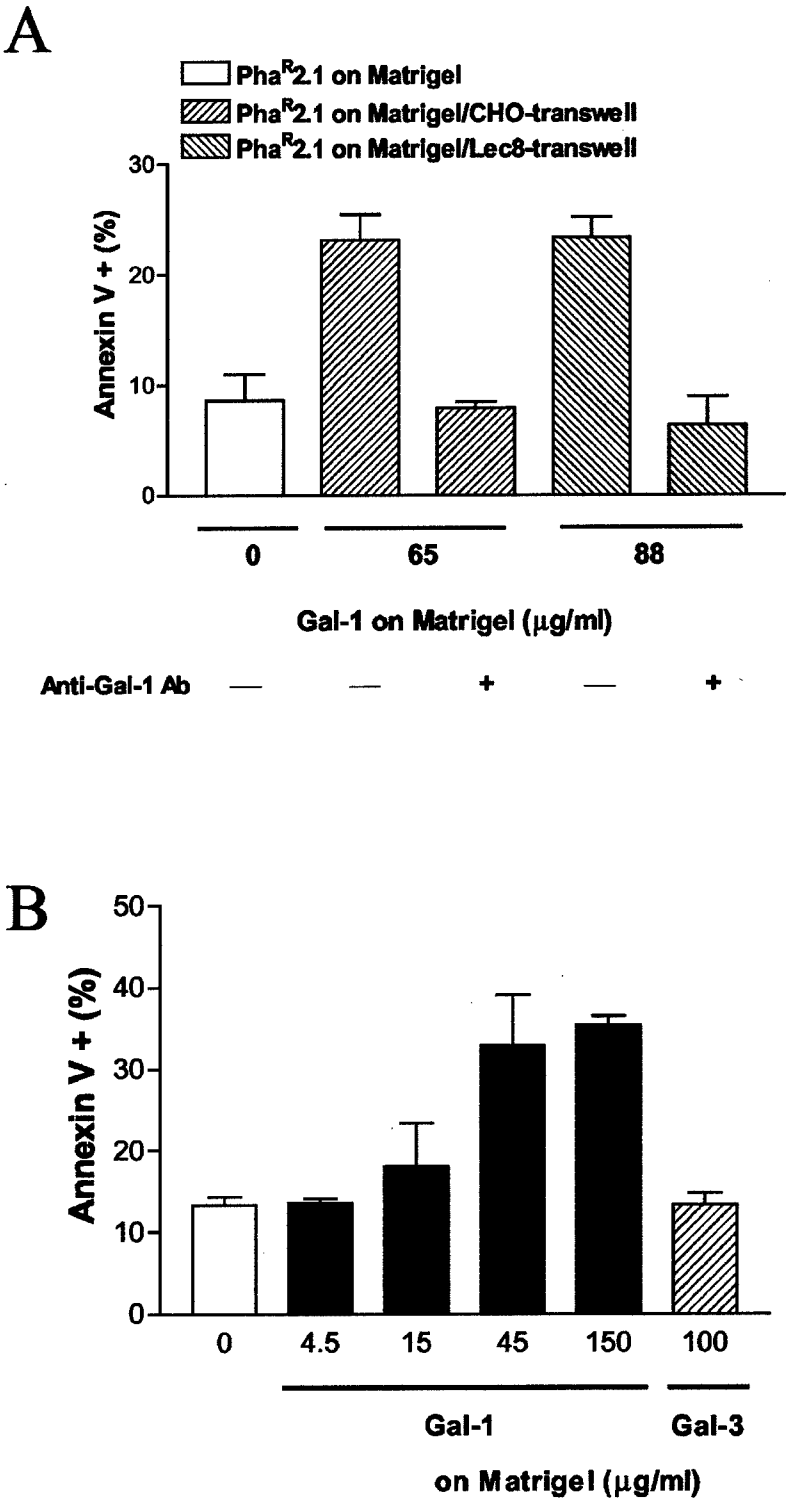


FIG. 4. **Galectin-1 on Matrigel is sufficient to kill T cells in the absence of stromal cells.** *A*, CHO, Lec8, or no stromal cells were placed in the upper well inserts over solidified Matrigel, in the bottom wells for 48 h. The galectin-1 concentration bound to the Matrigel from CHO or Lec8 cells was determined (*under* the respective *bars*). In parallel wells, 10^5 Pha^{R}2.1 cells were bound in the presence or absence of anti-gal-1 for 1 h, and the percent annexin V$^+$ T cells was calculated. *B*, indicated concentrations of galectin-1 or galectin-3 were bound directly to solidified Matrigel for 1 h. 10^5 Pha^{R}2.1 T cells were added to the Matrigel for 1 h, and % annexin V$^+$ T cells were determined. In *A* and *B*, values are the mean of triplicate samples.

1-coated Matrigel involved a brief T cell-stromal cell contact step before T cell adhesion to the matrix, we plated CHO or Lec8 cells in the upper wells of transwell plates with a layer of Matrigel in the bottom wells. Galectin-1 secreted by CHO or Lec8 cells diffused through the membrane in the upper chamber to deposit on the Matrigel (65 and 88 μg/ml, respectively, Fig. 4*A*). We examined the ability of galectin-1-coated Matrigel to kill adherent Pha^{R}2.1 T cells (Fig. 4*A*). Galectin-1 secreted by both CHO or Lec8 cells killed T cells bound to Matrigel. Of note, galectin-1 secreted from CHO or Lec8 cells killed the adherent T cells at surface concentrations of 2.6–3.5 μg/cm^2, or 65–88 μg/ml of Matrigel, a concentration of galectin-1 dramatically lower than the concentration of soluble galectin-1 required to kill the Pha^{R}2.1 T cells (Fig. 2*A*).

To determine the minimal amount of galectin-1 on Matrigel required to trigger T cell death, we coated solidified Matrigel with recombinant galectin-1. We observed T cell death when the amount of galectin-1 was as low as 1.7 μg/cm^2 (45 μg/ml) (Fig. 4*B*); in contrast, a comparable level of T cell death required ~500 μg/ml of soluble galectin-1 (Fig. 2*A*). As a control we examined the ability of galectin-3 to coat Matrigel and kill T cells; although galectin-3 bound to Matrigel, there was no

death of T cells on galectin-3-coated Matrigel at concentrations as high as 100 μg/ml (Fig. 4*B*).

These results demonstrate that T cell contact with galectin-1 either on the surface of another cell or on extracellular matrix in the absence of stromal cells can kill adherent T cells. Moreover, the amount of galectin-1 secreted by CHO and Lec8 cells was sufficient to kill adherent T cells when the galectin-1 was presented on the surface of the extracellular matrix, whereas we observed no T cell death when galectin-1 was secreted from CHO or Lec8 cell into media surrounding the T cells.

DISCUSSION

Galectin-1 participates in development, in immune system homeostasis, and in tumor progression (4–9). Pharmacologic administration of galectin-1 is effective in decreasing T cell responsiveness to antigens and inducing T cell apoptosis in several autoimmune disease models (9, 14); however, in these models immunosuppressive effects were not seen unless galectin-1 was administered at very high doses, typically 10 mg/kg, usually via an intraperitoneal route.

In contrast, although galectin-1 is very abundant in many cells and tissues (*e.g.* 35–40 μg/g of wet tissue in the spleen) (27), the serum concentration of galectin-1 in healthy women is only ~100 ng/ml (roughly equivalent to 420 μg of total serum galectin-1 in a 60-kg person), whereas patients with ovarian carcinoma (a type of tumor that expresses galectin-1 at high levels compared with normal tissue) have even lower serum levels of galectin-1, ~20 ng/ml (28). The reduced serum concentration of galectin-1 in ovarian carcinoma patients with tumors that synthesize high levels of galectin-1 may result from the increased deposition of galectin-1 on glycoproteins such as CA125 on the tumor cell surface as well as increased galectin-1 deposition in the ovarian carcinoma-associated stroma (29). The relatively low serum concentrations of galectin-1 in both healthy controls and cancer patients, in the ng/ml range, are far lower than the concentration of soluble, recombinant galectin-1 required for T cell death in *in vitro* assays (150 μg/ml). Thus, although galectin-1 has been called a cytokine (12), it is unlikely that increased galectin-1 synthesis in tumors would result in sufficiently elevated serum levels of galectin-1 to cause systemic immunosuppression.

However, as galectin-1 produced by tumors may primarily deposit on the tumor cells and surrounding stroma, galectin-1 may have profound local effects on the immune response to the tumor. Our data demonstrate that the acellular matrix can bind galectin-1 secreted from stromal cells and kill adherent T cells within 60 min. Although apoptotic death of tumor-infiltrating T cells has been observed, this has been attributed to T cell encounters with death ligands directly on the tumor cell surface (2) rather than in the tumor-associated stroma. Although the level of T cell death on the galectin-1-coated matrix (25–40%) was typically lower than the level of T cell death initiated by T cell-CHO cell contact (50–75%) (Figs. 3 and 4 *versus* Fig. 1), matrix-associated galectin-1 was sufficient to kill bound T cells; the membrane fluidity in cellular presentation of galectin-1 may facilitate the clustering of T cell glycoprotein counter-receptors that participate in galectin-1-induced cell death (21) compared with a more static presentation of galectin-1 on Matrigel.

Thus, the present study indicates that, even before encounter with tumor cells, T cells that bind galectin-1 in tumor-associated extracellular matrix may be triggered to die. As proposed by van den Brule and Castronovo (18), increased deposition of galectin-1 in carcinoma stroma may act as an "immunologic shield" surrounding tumor cells. As galectin-1 is secreted by many types of tumors, including glioblastoma, breast, and prostate carcinoma (4, 8), immunotherapy approaches in these cancers may be thwarted by the rapid death of T cells as they encounter galectin-1-loaded matrix surrounding the tumor. Moreover, galectin-1-induced phosphatidylserine exposure on leukocytes is sufficient for phagocytosis of the cells by macrophages (30), suggesting that stromal macrophages could rapidly eliminate infiltrating T cells that encounter galectin-1.

It is intriguing that joint tissue stroma from patients with rheumatoid arthritis had decreased galectin-1 deposition compared with normal joint tissues, in contrast to the increased galectin-1 deposition seen in tumor stroma (31). Thus, the T cell infiltration and immune-mediated damage in rheumatoid arthritis may relate to the decreased level of galectin-1 in joint stroma. Of interest, $CD4^+CD25^+$ regulatory T cells that express high levels of galectin-1 (13) typically require cell-cell contact to exert immunoregulatory function (3), consistent with our observation that galectin-1 bound to the cell surface or to the matrix is most effective at killing T cells.

The movement of galectin-1 from cells that produce it to the extracellular matrix is likely facilitated by the relatively low affinity (micromolar) of lectins for saccharide ligands, resulting in a rapid on-off rate on the surface of the cell (19, 32). However, the bivalency of galectin-1 may increase the likelihood of the secreted lectin remaining tethered to local glycoproteins, retaining the galectin-1 in the vicinity of cells that make it. Moreover, as the dissociation constant of the galectin-1 homodimer is in the micromolar range (19), the tethering of galectin-1 to matrix glycoproteins may increase the likelihood that the lectin remains in dimeric form, the form required to trigger T cell death. Of note, galectin-1 secreted from Lec8 cells will misfold and lose carbohydrate binding activity with a $t_{1/2}$ of ~10 h, whereas galectin-1 bound to saccharide ligands retains binding activity for weeks (19, 23). Galectin-1 secreted from Lec8 cells onto Matrigel over 24–72 h retained binding activity, suggesting that the secreted lectin bound fairly rapidly to saccharide ligands in the Matrigel. In addition, a galectin-1 concentration as low as 45 μg/ml was sufficient to kill T cells when the galectin-1 was presented on the Matrigel surface, whereas a 10-fold higher concentration of soluble galectin-1 was required to kill an equivalent fraction of T cells.

Extracellular matrix glycoproteins may, thus, serve three roles in mediating galectin-1-triggered T cell death. First, the extracellular matrix provides a rich source of saccharide ligands on glycoproteins such as laminin, fibronectin, and vitronectin, which can contribute to maintaining galectin-1 carbohydrate binding activity. Second, matrix glycoproteins can concentrate galectin-1 secreted by surrounding cells to increase the fraction of galectin-1 molecules that are homodimers. Third, the matrix presents galectin-1 to the T cell surface in a two-dimensional array rather than in a three-dimensional space as when T cells encounter soluble galectin-1; this type of presentation may facilitate the interaction of galectin-1 with the large T cell surface glycoproteins CD43 and CD45 that form a glycocalyx surrounding the T cell to increase the rate and/or duration of galectin-1 binding to the cell (32). Understanding the mechanism by which galectin-1 interacts with extracellular matrix in tissues is critical for designing effective therapeutic strategies for galectin-1 in localized and systemic autoimmune disease and in manipulating the immune response to tumors.

Acknowledgments—We thank Leland D. Powell and Karen Pace for advice and review, Sorge Kelm and Nancy Perillo for insights, and Mabel Pang for technical assistance.

REFERENCES

1. Palmer, E. (2003) *Nat. Rev. Immunol.* **3**, 383–391
2. Rivoltini, L., Carrabba, M., Huber, V., Castelli, C., Novellino, L., Dalerba, P.,

Mortarini, R., Arancia, G., Anichini, A., Fais, S., and Parmiani, G. (2002) *Immunol. Rev.* **188,** 97–113
3. McHugh, R. S., and Shevach, E. M. (2002) *J. Allergy Clin. Immunol.* **110,** 693–702
4. Perillo, N. L., Marcus, M. E., and Baum, L. G. (1998) *J. Mol. Med* **76,** 402–412
5. Hughes, R. C. (2001) *Biochimie (Paris)* **83,** 667–676
6. Rabinovich, G. A., Baum, L. G., Tinari, N., Paganelli, R., Natoli, C., Liu, F. T., and Iacobelli, S. (2002) *Trends Immunol.* **23,** 313–320
7. Liu, F. T., Patterson, R. J., and Wang, J. L. (2002) *Biochim. Biophys. Acta* **1572,** 263–273
8. Danguy, A., Camby, I., and Kiss, R. (2002) *Biochim. Biophys. Acta* **1572,** 285–293
9. Hernandez, J. D., and Baum, L. G. (2002) *Glycobiology* **12,** 127–136
10. Almkvist, J., Dahlgren, C., Leffler, H., and Karlsson, A. (2002) *J. Immunol.* **168,** 4034–4041
11. Correa, S. G., Sotomayor, C. E., Aoki, M. P., Maldonado, C. A., and Rabinovich, G. A. (2003) *Glycobiology* **13,** 119–128
12. Blaser, C., Kaufmann, M., Muller, C., Zimmermann, C., Wells, V., Mallucci, L., and Pircher, H. (1998) *Eur. J. Immunol.* **28,** 2311–2319
13. McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M., and Byrne, M. C. (2002) *Immunity* **16,** 311–323
14. Santucci, L., Fiorucci, S., Rubinstein, N., Mencarelli, A., Palazzetti, B., Federici, B., Rabinovich, G. A., and Morelli, A. (2003) *Gastroenterology* **124,** 1381–1394
15. Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995) *Nature* **378,** 736–739
16. Gauthier, L., Rossi, B., Roux, F., Termine, E., and Schiff, C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13014–13019
17. van den Brule, F., Califice, S., Garnier, F., Fernandez, P. L., Berchuck, A., and Castronovo, V. (2003) *Lab. Invest.* **83,** 377–386
18. van den Brule, F. A., Waltregny, D., and Castronovo, V. (2001) *J. Pathol.* **193,** 80–87
19. Cho, M., and Cummings, R. D. (1995) *J. Biol. Chem.* **270,** 5198–5206
20. Nguyen, J. T., Evans, D. P., Galvan, M., Pace, K. E., Leitenberg, D., Bui, T. N., and Baum, L. G. (2001) *J. Immunol.* **167,** 5697–5707
21. Amano, M., Galvan, M., He, J., and Baum, L. G. (2003) *J. Biol. Chem.* **278,** 7469–7475
22. Rabinovich, G. A., Iglesias, M. M., Modesti, N. M., Castagna, L. F., Wolfenstein-Todel, C., Riera, C. M., and Sotomayor, C. E. (1998) *J. Immunol.* **160,** 4831–4840
23. Cho, M., and Cummings, R. D. (1995) *J. Biol. Chem.* **270,** 5207–5212
24. Stanley, P., Sundaram, S., and Sallustio, S. (1991) *Glycobiology* **1,** 307–314
25. Fukaya, K., Hasegawa, M., Mashitani, T., Kadoya, T., Horie, H., Hayashi, Y., Fujisawa, H., Tachibana, O., Kida, S., and Yamashita, J. (2003) *J. Neuropathol. Exp. Neurol.* **62,** 162–172
26. Maeda, N., Kawada, N., Seki, S., Arakawa, T., Ikeda, K., Iwao, H., Okuyama, H., Hirabayashi, J., Kasai, K., and Yoshizato, K. (2003) *J. Biol. Chem.* **278,** 18938–18944
27. Ahmed, H., Fink, N. E., Pohl, J., and Vasta, G. R. (1996) *J. Biochem. (Tokyo)* **120,** 1007–1019
28. Allen, H. J., Sharma, A., Ahmed, H., Piver, M. S., and Gamarra, M. (1993) *Tumour Biol.* **14,** 360–368
29. Seelenmeyer, C., Wegehingel, S., Lechner, J., and Nickel, W. (2003) *J. Cell Sci.* **116,** 1305–1318
30. Dias-Baruffi, M., Zhu, H., Cho, M., Karmakar, S., McEver, R. P., and Cummings, R. D. (2003) *J. Biol. Chem.* **278,** 41282–41293
31. Harjacek, M., Diaz-Cano, S., De Miguel, M., Wolfe, H., Maldonado, C. A., and Rabinovich, G. A. (2001) *J. Rheumatol.* **28,** 1914–1922
32. Brewer, C. F., Miceli, M. C., and Baum, L. G. (2002) *Curr. Opin. Struct. Biol.* **12,** 616–623

Clusters, bundles, arrays and lattices: novel mechanisms for lectin–saccharide-mediated cellular interactions

C Fred Brewer*, M Carrie Miceli† and Linda G Baum‡

Multivalent protein–carbohydrate interactions regulate essential cellular events, including cell proliferation, adhesion and death. These multivalent interactions can create homogeneous complexes of lectins, such as the galectins, with their saccharide ligands. Lectin–saccharide complexes can concentrate specific glycoproteins or glycolipids within the lattice, while excluding other cell surface molecules. The formation of lectin–saccharide lattices on the cell surface can thus organize the plasma membrane into specialized domains that perform unique functions.

Addresses
*Departments of Molecular Pharmacology, Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA
†Department of Microbiology, Immunology and Molecular Genetics, and Jonsson Comprehensive Cancer Center and Molecular Biology Institute, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
‡Department of Pathology and Laboratory Medicine, and Jonsson Comprehensive Cancer Center and Molecular Biology Institute, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA; e-mail: lbaum@mednet.ucla.edu

Current Opinion in Structural Biology 2002, **12**:616–623

0959-440X/02/$ – see front matter

Abbreviations

CRD	carbohydrate recognition domain
ECM	extracellular matrix
Gal	galactose
GlcNAc	*N*-acetylglucosamine
MHC	major histocompatibility complex
TCR	T-cell receptor

Introduction

Cells are in constant communication with their surroundings. For normal and pathological processes, including cell proliferation, organ morphogenesis, inflammation, wound healing and tumor metastasis, cells must be able to receive signals from the extracellular milieu and deliver those signals to the inside of the cell. In the past decade, the field of signal transduction has made enormous advances in understanding how 'outside-in' signals are transduced and how those signals are translated inside the cell. However, much of this work has examined protein–protein interactions, that is, the binding of an extracellular protein ligand to a protein receptor on the cell surface, to deliver information from the extracellular milieu to the cell.

Recent work demonstrates that carbohydrate–protein interactions are also critical triggers in cell signaling [1,2•,3,4••,5–7]. These interactions involve the binding of lectins, or carbohydrate recognition proteins, to specific saccharide ligands. Signaling can occur when cell surface lectins transduce a signal after binding saccharide ligands; for example, the B cell lectin CD22 binding to its ligand sialic acid regulates the strength of immunoglobulin signaling [8•,9•]. Signaling can also occur when soluble lectins bind to carbohydrates on cell surface glycoproteins or glycolipids; for example, binding of soluble galectin-1 to its ligand *N*-acetyllactosamine on T cell surface glycoproteins triggers the T cells to die [10–12].

Compared with protein–protein interactions, carbohydrate–protein interactions have several novel features. First, these interactions are regulated at the genetic level by the expression of glycosyltransferase enzymes that create the carbohydrate ligands [4••,11], rather than the direct expression of genes for ligands or receptors. Second, the glycosyltransferases that create a carbohydrate ligand may add that ligand to several different protein backbones on the cell surface. Third, glycoproteins often bear multiple copies of the saccharide ligands that are recognized by lectins [13••,14], either as repeating units on a single oligosaccharide, as seen with mannose residues on yeast glycoproteins [15], or as clustered repeats of the saccharide ligand on the protein backbone, as seen with receptors for the selectins [16]. The lectins that bind the saccharide ligands are often multivalent as well. The multivalent nature of both lectins and their saccharide ligands allows the formation of a lectin–carbohydrate lattice, which acts as a signaling complex at the cell surface.

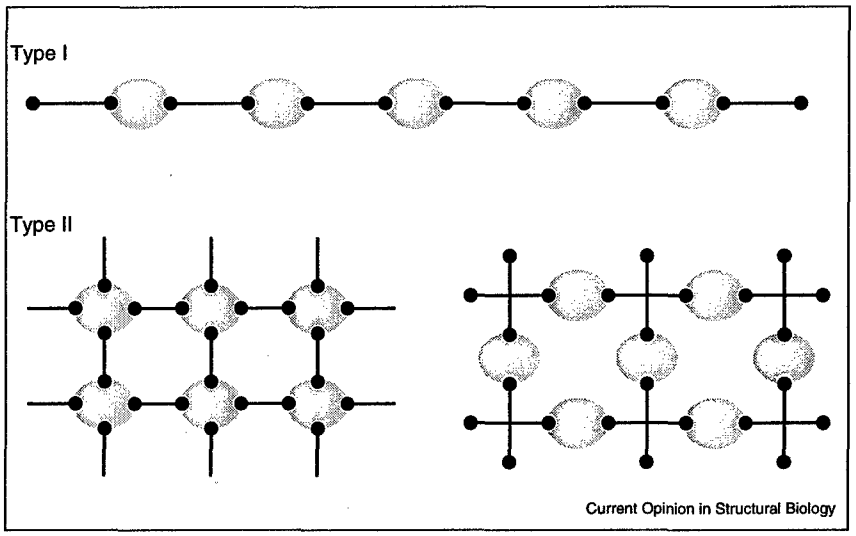
Lectin–saccharide interactions

Lectin binding to a single saccharide ligand is typically a low-affinity interaction. However, the multivalent nature of lectin–saccharide interactions allows many low-affinity binding events to occur, resulting in high overall avidity [7,17,18]. This multivalency or 'glycoside cluster' effect has been described for several multivalent mammalian lectins. For example, the serum mannose-binding protein preferentially recognizes clustered mannose residues [15] and dimerization of L-selectin increases leukocyte adhesion to clustered saccharide ligands [19,20]. The galectins are a growing family of multivalent lectins [1,7]; several groups have shown that galectins preferentially bind to glycans carrying repeating units of the ligand *N*-acetyllactosamine (Galβ1,4GlcNAc), either as disaccharide units at the termini of tri- or tetra-antennary chains on *N*-glycans, or as repeating units in a poly-*N*-acetyllactosamine chain on *N*- or *O*-glycans [18,21–24].

Multimeric lectins can cross-link multivalent carbohydrate ligands. Given the abundance of saccharides on cell surface glycoproteins and glycolipids, and the ability of glycosyltransferases to add the same glycan structure to

Figure 1

Lectin–saccharide lattices. Type I complexes are composed of bivalent lectins and bivalent carbohydrates. These polymers can be flexible and can accommodate carbohydrate ligands of different lengths. Type II complexes are composed of lectins and carbohydrates, one of which has a valency >2. On the left, a tetravalent lectin is complexed to a bivalent carbohydrate, whereas on the right a bivalent lectin is complexed with a tetravalent carbohydrate. Type II complexes favor inclusion of a single species of carbohydrate ligand, which facilitates the formation of thermodynamically favorable lattices.



different protein or lipid backbones, one might imagine that lectin–saccharide cross-linking could result in one big heterogeneous complex on the cell surface. Surprisingly, several studies with galectins and other lectins have shown that, in contrast to agglutinating a heterogeneous mix of glycoprotein ligands, multivalent lectins selectively cross-link a single species of glycoprotein to form uniform lectin–carbohydrate lattices [3,12,25].

Lectin–carbohydrate interactions can occur in two ways: type I and type II [3] (Figure 1). Type I complexes are formed by bivalent lectins and bivalent ligands, to form linear polymers. These polymers may be polymorphic, because the linear arrangement tolerates flexibility and the ligands can be of different lengths and composition, as long as they are bivalent. By contrast, type II complexes are formed when either the lectin or the ligand has a valency >2. In this case, ordered cross-linked complexes are formed that are homogeneous with respect to ligand, even when several potential ligand species, that is, glycoproteins, are in the mix. The regular spacing in the lattice conferred by the repeating use of a single ligand species is thermodynamically favorable, as shown by studies with a variety of multivalent lectins, including galectins [3,18].

The multivalent galectins

The galectins are a family of lectins defined by a conserved carbohydrate recognition domain (CRD), found in species ranging from fungi to human. Fourteen mammalian galectins have been identified. The structures and biological activities of many of the galectins are the subject of recent excellent reviews [1,7,26] and so will not be examined comprehensively here.

Importantly, all mammalian galectins can act as multivalent lectins. Some galectins, including galectin-1, are synthesized as monomers with a single CRD; these galectins typically dimerize through noncovalent interactions to create functionally bivalent lectins. The tandem-repeat galectins have two CRDs connected by a linker peptide and are thus bivalent, although the two CRDs may be able to recognize slightly different saccharide ligands. Galectin-3 is a chimeric galectin with a C-terminal CRD attached to an N-terminal peptide. However, galectin-3 can also be multimeric, as it can spontaneously form multimers on the cell surface or can be cross-linked by tissue transglutaminase [7].

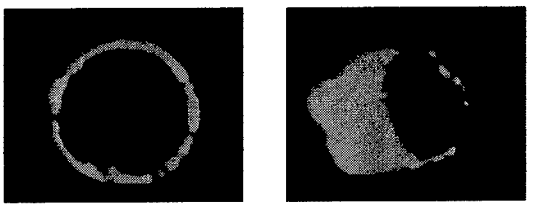
Galectins have a variety of functions in specific tissues and can induce cell proliferation, cell cycle arrest or cell death. Galectins have been implicated in organ morphogenesis, tumor cell metastasis and leukocyte trafficking — all processes that involve adhesion and de-adhesion of different cell types, as well as the recognition of adjacent cells and the extracellular matrix (ECM). Although the *N*-acetyllactosamine ligand recognized by galectins is expressed ubiquitously on *N*- and *O*-linked glycans, galectins can discriminate among different *N*-acetyllactosamine-bearing glycoproteins, which suggests that the preferential recognition of specific *N*-acetyllactosamine-bearing glycoproteins is important for conferring unique galectin functions in specific cell types.

In solution, galectins can discriminate among different multivalent glycoprotein ligands to form homogenous lattices of lectin and glycoprotein [18]. On the cell surface, galectins selectively recognize different glycoprotein ligands [7,25]. This suggests that, on the cell surface, galectins may form homogenous lattices with specific glycoproteins.

Galectin-mediated T cell signaling: lattice control?

In the immune system, programmed cell death is a critical mechanism that avoids the development of autoreactive T cells and limits the extent of an immune response.

Figure 2



Galectin-1-induced clustering of the T cell surface glycoprotein receptor CD45. Before galectin-1 binding, CD45 is distributed uniformly over the surface of the T cell (left). After galectin-1 binding, CD45 is clustered onto membrane 'blebs' that exclude other cell surface glycoproteins (right). Localization of CD45 was detected with a fluorescein-labeled antibody to CD45 and cells were analyzed by confocal microscopy.

Galectin-1, widely expressed in a variety of organs, induces death of developing T cells in the thymus and activated T cells in the periphery [10,27,28]. Several T cell glycoproteins that can bind galectin-1 have been identified, including CD3, CD7, CD43 and CD45 [25,29,30]. This suggested that either galectin-1 bound all of these receptors in a heterogeneous complex on the cell surface or galectin-1 selectively cross-linked specific receptors into a homogeneous signaling lattice, analogous to the lattice formation observed in solution.

To examine these two possibilities, the surface distribution of CD3, CD7, CD43 and CD45 on T cells was examined before and after galectin-1 binding [25]. Pace *et al.* [25] found that galectin-1 selectively forms complexes containing either CD45 and CD3, or CD7 and CD43, resulting in the segregation of CD3/CD45 from CD7/CD43 complexes on the cell surface (Figure 2). Segregation of CD45 from CD7 and CD43 appeared to be essential to initiate cell death; the CD45 cytoplasmic domain has tyrosine phosphatase activity that could antagonize pro-death signals delivered via CD7/CD43, so that the segregation of CD45 may allow the death signal to be delivered unopposed [12]. These studies demonstrated that homotypic lattices of galectin and specific glycoproteins can form on the cell surface and that these lattices regulate the signal delivered by galectin-1 binding.

Galectin-1-mediated lattice formation can also influence signals sent by other receptor–ligand systems. Engagement of the T-cell receptor (TCR) plus a co-stimulatory molecule either by antigen presentation or by cross-linking antibodies causes clustering of TCRs that signal the cell to proliferate and to secrete cytokines [31]. Galectin-1 binding to T cells antagonizes this signal by limiting the clustering of membrane microdomains containing the TCRs after engagement [32] (Figure 3). In this manner, galectin-1 binding to TCRs and/or associated glycoproteins appears to keep the TCRs spaced a critical distance apart on the cell surface, thus acting as a brake on the amplitude, processivity and/or duration of signaling by the TCR.

A similar effect has been ascribed to galectin-3. Dennis and co-workers [4••,5] examined mice lacking the GnT V enzyme, which can create multivalent poly-*N*-acetyllactosamine oligosaccharides that are preferentially recognized by galectins. T cells from the GnT V$^{-/-}$ mice demonstrated enhanced TCR responses, with dramatic TCR clustering after TCR engagement, which suggests that the GnT V enzyme participates in creating the cell surface structures that normally limit the extent of TCR interactions. This effect could be phenocopied by pre-incubating the cells with lactose to dissociate bound galectin-3, suggesting that galectin-3 mediates the GnT-V-dependent negative regulation of T cell signaling. Thus, galectins can act as a brake on TCR signaling, by forming a lattice with specific glycans on TCR glycoproteins to limit the clustering of TCRs after antigen binding.

Galectins and cell adhesion: bundles and clusters

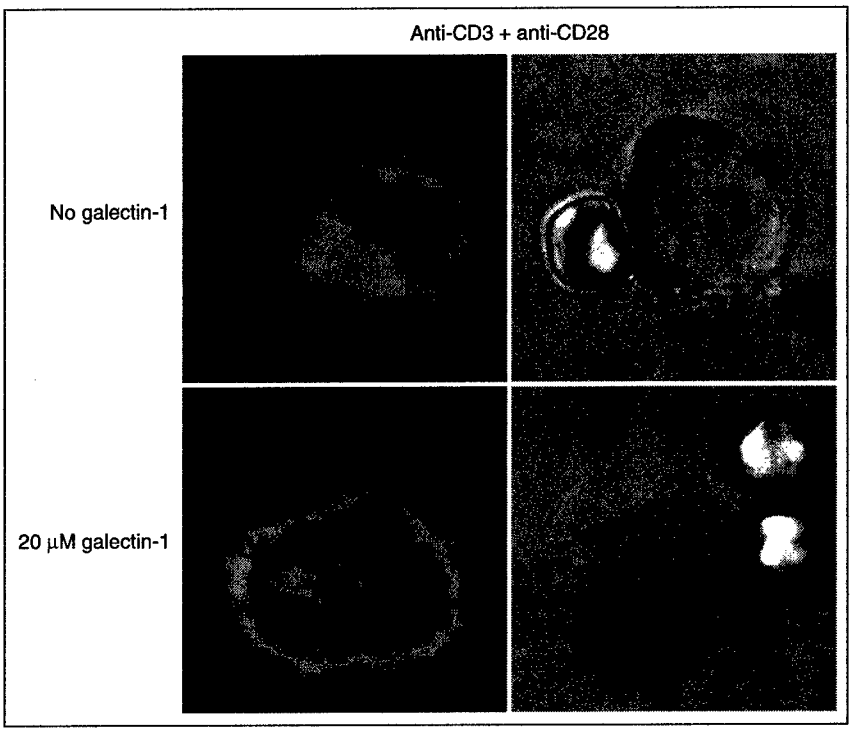
As described above, galectins control T cell fate both directly by triggering cell death and indirectly by modulating signals sent through other signaling pathways, such as that of the TCR. A large body of work has examined the roles of galectins in mediating cell adhesion to the ECM, an important process in organogenesis and tumor metastasis (reviewed in [7]). Strikingly, there are again examples showing that galectins directly mediate cell adhesion and also indirectly modulate cell adhesion through other molecules, such as integrins. In both situations, galectins appear to interact with multivalent saccharide ligands, suggesting that formation of the lectin–saccharide lattice is also involved in cell adhesion.

Galectins can bind directly to ECM proteins, such as laminin, that display abundant poly-*N*-acetyllactosamine-containing glycans on the polypeptide backbone [21]. Dimeric galectin-1 directly mediated the binding of smooth muscle cells and melanoma cells to laminin. In contrast, galectin-3 at a low concentration did not mediate melanoma cell–laminin binding; however, after transglutaminase cross-linking, multivalent galectin-3 could mediate melanoma cell–ECM binding, indicating that multivalent lectin binding was critical for adhesion. Galectin-3 also mediates neutrophil adhesion to laminin and, at low concentrations, the adhesion of breast cancer cells to the ECM (reviewed in [7]).

Galectin-1 and galectin-3 can both indirectly influence cell adhesion as well. Galectin-1 antagonizes lymphocyte adhesion to integrins [33], whereas high concentrations of galectin-3 antagonize breast cancer cell adhesion to integrins [34•]. Galectin-3 secreted from epithelial cells has also been found to 'bundle' or polymerize the ECM protein hensin [35]. In the presence of galectin-3, hensin is polymerized and causes terminal differentiation of

Figure 3

Galectin-1 binding antagonizes lipid raft reorganization. In the absence of galectin-1, T cell binding to beads coated with antibodies to CD3 and CD28 results in the relocalization of lipid rafts to the site of engagement (top). In the presence of galectin-1, lipid raft reorganization is prevented and rafts do not cluster at the cell–bead interface (bottom). Lipid rafts were visualized with a green fluorescent protein targeted specifically to lipid rafts by fusion to the N-terminal ten amino acids of the intracellular kinase lck (left) and bright field images of the same cell are shown (right). Reproduced with permission from [32].



epithelial cells, whereas in the absence of galectin-3, hensin remains unpolymerized and has no differentiation effect. Thus, galectin-3 modulates the ECM signal mediated by hensin to direct cell fate.

In addition, galectin-8, a tandem-repeat galectin with two CRDs, has both pro-adhesive and anti-adhesive functions [36]. By binding to cell surface integrins, immobilized galectin-8 can directly mediate the adhesion and spreading of various cell types. But neither truncated galectin-8 with only one CRD nor excess soluble galectin-8 mediates cell adhesion and spreading, which suggests that the physical arrangement of the galectin-8–integrin interactions is important for cell adhesion and spreading, rather than simply the engagement of cell surface integrins.

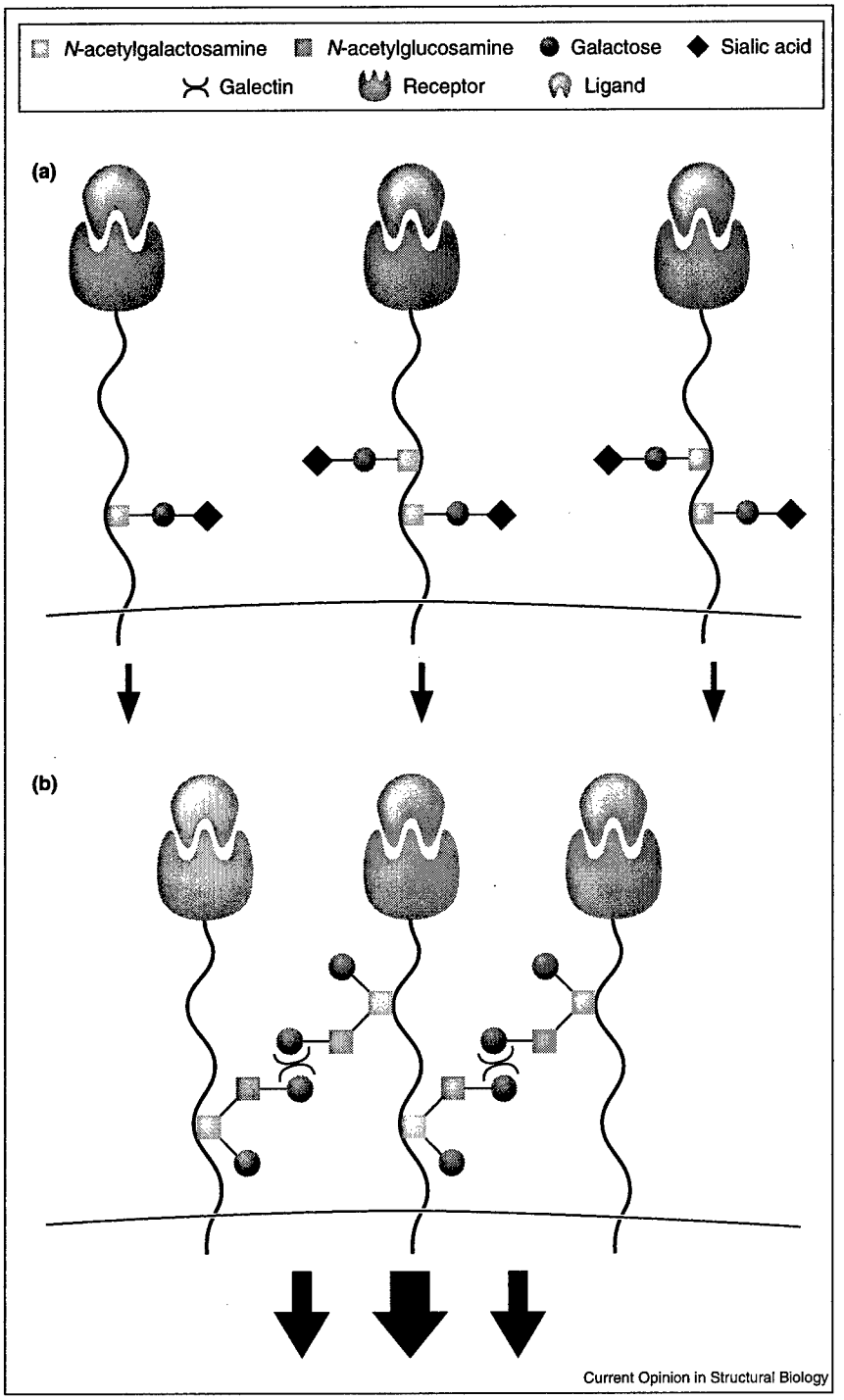
Galectin localization in membrane microdomains: floating in flasks and rafts

The ability of galectins to organize membrane glycoproteins into discrete microdomains on the cell surface suggests that the multivalent lectin–saccharide interactions occur preferentially in these specific domains. A type of lipid microdomain that seems to be essential for assembling signal transduction components at the plasma membrane is the lipid raft, a glycolipid-enriched membrane domain [31,37]. Lipid rafts concentrate specific membrane glycoproteins, glycolipids and cytosolic signaling proteins in order to modulate signals produced when extracellular ligands engage cell surface receptors. For example, lipid rafts containing TCRs coalesce after the TCRs bind antigen on antigen-presenting cells expressing co-stimulatory ligands.

One mechanism by which galectin-1 modulates TCR signaling is by limiting the coalescence of lipid rafts and thus the extent of TCR phosphorylation [32]. Indeed, galectin-1 binding to T cells results in partial but incomplete phosphorylation of the TCR ζ chain. In addition, disrupting lipid raft integrity with the cholesterol-chelating agent methyl-β-cyclodextrin inhibits galectin-1-induced ζ chain partial phosphorylation, implying that lipid rafts are required for galectin-1-mediated effects. Lipid rafts are also rich in the ganglioside GM1 [37] and antibodies to GM1 inhibit galectin-1 binding to neuroblastoma cells, suggesting that cell surface glycoproteins recognized by galectin-1 are concentrated in lipid rafts [38]. Collectively, these data indicate that galectin-1 can selectively associate with specific glycoproteins in lipid raft membrane microdomains.

Intriguing new work also localizes galectin-3 to lipid rafts. As described above, galectin-3 can antagonize the integrin-mediated adhesion of breast cancer cells. Ochieng and co-workers [34•] have found recently that this process occurs in part by the galectin-3-mediated internalization of cell surface integrins into caveolae, flask-shaped invaginations that dip down from lipid rafts to allow clathrin-independent endocytosis. In many cell types, integrins may be excluded from rafts, unless specific cell activation events occur [39,40]. This implies that

Figure 4



Model of galectin-mediated glycoprotein lattices enhancing signaling by an unrelated ligand. **(a)** Individual receptors engage individual ligands and each sends a weak signal. The presence of sialic acid inhibits galectin-mediated cross-linking of the receptors. **(b)** Appropriate glycosylation allows galectin-mediated cross-linking of the receptors. Subsequent engagement by ligands sends signals that are temporally and spatially concentrated to result in an overall increase in signal strength and/or duration.

galectin-3 binding first concentrated and localized cell surface integrins into lipid rafts, prior to caveolae-mediated internalization.

In intestinal epithelial cells, endogenous galectin-4 is defined as a marker of membrane lipid rafts [41]. On these cells, galectin-4 binds to two cell surface brush border enzymes and remains associated with the lipid raft membrane microdomains. Because galectins, including galectin-4, are secreted by a nonclassical secretion pathway and thus cannot associate intracellularly with the brush border enzymes before externalization from the cell [42,43], the association of galectin-4 with specific brush border enzymes in lipid rafts again demonstrates a

preferential recognition of glycoprotein ligands on the cell surface, resulting in the formation of galectin–glycoprotein clusters in specific membrane domains.

Lectin–saccharide interactions: priming the system

Three possible functions for multivalent galectin–saccharide lattices have been described above. First, these interactions can directly signal a specific cell event, such as galectin-1-induced cell death. Second, these interactions can act as a rheostat on other types of signaling events, such as galectin-1 and galectin-3 antagonism of TCR signaling. Third, these interactions can concentrate cell surface glycoproteins into specific membrane domains to allow domain-specific events to occur, such as the galectin-3-mediated endocytosis of integrins via caveolae. A fourth possible function for galectin–saccharide lattice interactions has been suggested by recent work demonstrating the effect of T cell surface glycosylation on T cell development in the thymus [44••,45••]. This work implies that carbohydrate-mediated interactions of T cell surface glycoproteins potentiate the ability of these glycoproteins to bind their cognate protein receptors.

During T cell maturation in the thymus, specific glycosyltransferase enzymes are expressed at precise points during T cell development. Recently, Moody *et al.* [44••] and Daniels *et al.* [45••] have found that expression of a specific sialyltransferase, ST3Gal I, increases cell surface sialylation and reduces the ability of the CD8 antigen carried on developing T cells to bind major histocompatibility complex (MHC) class I molecules. The binding of the MHC class I protein complex to CD8 is a well-characterized protein–protein interaction and MHC recognition is an important step in proper T cell maturation that allows T cells in the periphery to recognize foreign antigens. Expression of ST3Gal I can also reduce galectin-1 binding to T cell surface glycoproteins, by competing for substrate with the branching enzyme that catalyzes the extension of poly-*N*-acetyllactosamine chains that are preferentially recognized by galectin-1 [11]. This suggests that, in the absence of sialylation, the cross-linking and clustering of cell surface glycoproteins mediated by galectin-1 may enhance the binding of MHC class I molecules to CD8 antigens on developing T cells [46]. There may be other systems in which galectin-mediated cross-linking and clustering of cell surface glycoproteins 'organize' receptors on the cell surface to enhance ligand binding and to increase the amplitude of subsequent intracellular signals (Figure 4).

Conclusions

Traditionally, the transmission of outside-in signals to a cell has been envisaged as a bimolecular interaction in which a protein ligand binds a protein receptor that sends a signal into the cell. It is now becoming clear that, for many systems, the clustering of protein receptors and ligands into a 'synapse' is required for optimal signaling [31], although the forces driving that clustering are still poorly understood [47,48]. However, the model of multivalent protein–carbohydrate interactions discussed here involves the assembly of ordered arrays of lectins and saccharides into large, discrete domains on the cell surface. We therefore propose that the assembly of these arrays, a thermodynamically favorable process, drives subsequent cellular events such as proliferation, adhesion or death. Physiologically, a cell may be covered with endogenous lectins binding to saccharide ligands to create local associations of specific glycoproteins and glycolipids in specific membrane domains. These associations, which are made up of several low-affinity interactions, may be potentiated or antagonized by the engagement of additional signaling molecules. The variables that govern assembly of these lattices might include branching of saccharide ligands [4••,11], presentation of the ligands along protein or lipid backbones [16–23], and the spacing distance of the binding domains of multivalent lectins [49]. Recent work has shown that this type of lectin–saccharide clustering occurs during the interaction of myelin-associated glycoprotein, a lectin, with the glycolipid receptors, GD1a and GT1b, on nerve cells — a process that triggers neurite outgrowth and that may be modulated by other signaling pathways [50]. Given that virtually every protein on the surface of a cell is glycosylated, lectin–saccharide lattices may be ubiquitous participants in all types of cellular communication.

Acknowledgements

We thank members of the Brewer, Miceli and Baum laboratories for contributions to the work described here, and LD Powell for critically reading the manuscript. Supported in part by National Institutes of Health (NIH) grant GM63281, Department of Defense grant 17-02-1-0022 and a Cancer Research Institute grant to LG Baum, NIH grant CA16054 to CF Brewer, and NIH grant CA65979 and an Arthritis Foundation Research Grant to MC Miceli.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Rabinovich GA, Baum LG, Tinari N, Paganelli R, Natoli C, Liu F-T, Iacobelli S: **Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response?** *Trends Immunol* 2002, **23**:313-320.

2. • Hakomori S: **The glycosynapse.** *Proc Natl Acad Sci USA* 2002, **99**:225-232.
This inaugural article summarizes current evidence that many different glycoproteins and glycolipids participate in organizing the plasma membrane into functionally distinct microdomains that regulate functions such as adhesion and cell–cell signaling.

3. Sacchettini JC, Baum LG, Brewer CF: **Multivalent protein–carbohydrate interactions. A new paradigm for supermolecular assembly and signal transduction.** *Biochemistry* 2001, **40**:3009-3015.

4. •• Demetriou M, Granovsky M, Quaggin S, Dennis JW: **Negative regulation of T-cell activation and autoimmunity by Mgat5 *N*-glycosylation.** *Nature* 2001, **409**:733-739.
Mice lacking the GnT V enzyme (*Mgat5*$^{-/-}$) show increased hypersensitivity to T cells and enhanced development of autoimmunity. Loss of the poly-*N*-acetyllactosamine oligosaccharide branches created by GnT V results in reduced galectin-3 binding, which shows that glycoprotein–galectin lattices on the cell surface modulate TCR signaling.

5. Dennis JW, Warren CE, Granovsky M, Demetriou M: **Genetic defects in *N*-glycosylation and cellular diversity in mammals.** *Curr Opin Struct Biol* 2001, **11**:601-607.

6. Lowe JB: **Glycosylation, immunity, and autoimmunity.** *Cell* 2001, **104**:809-812.

7. Hughes RC: **Galectins as modulators of cell adhesion.** *Biochimie* 2001, **83**:667-676.

8. • Kelm S, Gerlach J, Brossmer R, Danzer CP, Nitschke L: **The ligand-binding domain of CD22 is needed for inhibition of the B cell receptor signal, as demonstrated by a novel human CD22-specific inhibitor compound.** *J Exp Med* 2002, **195**:1207-1213.
See annotation to [9•].

9. • Jin L, McLean PA, Neel BG, Wortis HH: **Sialic acid binding domains of CD22 are required for negative regulation of B cell receptor signaling.** *J Exp Med* 2002, **195**:1199-1205.
These two papers [8•,9•] show that ligand engagement by a transmembrane B cell lectin results in altered B cell signaling in response to immunoglobulin engagement. This work directly links saccharide recognition by the extracellular lectin domain to signaling by the intracellular domain.

10. Perillo NL, Pace KE, Seilhamer JJ, Baum LG: **Apoptosis of T cells mediated by galectin-1.** *Nature* 1995, **378**:736-739.

11. Galvan M, Tsuboi S, Fukuda M, Baum LG: **Expression of a specific glycosyltransferase enzyme regulates T cell death mediated by galectin-1.** *J Biol Chem* 2000, **275**:16730-16737.

12. Nguyen JT, Evans DP, Galvan M, Pace KE, Leitenberg D, Bui TN, Baum LG: **CD45 modulates galectin-1 induced cell death: regulation by expression of core 2 *O*-glycans.** *J Immunol* 2001, **167**:5697-5707.

13. •• Rudd PM, Elliot T, Cresswell P, Wilson IA, Dwek RA: **Glycosylation and the immune system.** *Science* 2001, **291**:2370-2375.
An excellent synthesis of the various ways in which glycosylation, both within a cell and on its surface, controls protein folding and transport, antigen recognition and presentation, and the interaction of antigen receptors in the 'immune synapse', stressing the effects that glycosylation can have on immune signaling. This review and the one by Bertozzi and Kiessling [14] are part of a special feature on carbohydrates and glycobiology in *Science*.

14. Bertozzi CR, Kiessling LL: **Chemical glycobiology.** *Science* 2001, **291**:2357-2364.

15. Lee RT, Lee YC: **Affinity enhancement by multivalent lectin–carbohydrate interaction.** *Glycoconj J* 2000, **17**:543-551.

16. Norgard KE, Moore KL, Diaz S, Stults NL, Ushiyama S, McEver RP, Cummings RD, Varki A: **Characterization of a specific ligand for P-selection on myeloid cells. A minor glycoprotein with sialylated *O*-linked oligosaccharides.** *J Biol Chem* 1993, **268**:12764-12774.

17. Dam TK, Roy R, Das SK, Oscarson S, Brewer CF: **Binding of multivalent carbohydrates to concanavalin A and *Dioclea grandiflora* lectin. Thermodynamic analysis of the "multivalency effect".** *J Biol Chem* 2000, **275**:14223-14230.

18. Gupta D, Kaltner H, Dong X, Gabius HJ, Brewer CF: **Comparative cross-linking activities of lactose-specific plant and animal lectins and a natural lactose-binding immunoglobulin G fraction from human serum with asialofetuin.** *Glycobiology* 1996, **6**:843-849.

19. Ramachandran V, Yago T, Epperson TK, Kobzdej MM, Nollert MU, Cummings RD, Zhu C, McEver R: **Dimerization of a selectin and its ligand stabilizes cell rolling and enhances tether strength in shear flow.** *Proc Natl Acad Sci USA* 2001, **98**:10166-10171.

20. Dwir O, Steeber DA, Schwarz US, Camphausen RT, Kansas GS, Tedder TF, Alon R: **L-selectin dimerization enhances tether formation to properly spaced ligand.** *J Biol Chem* 2002, **277**:21130-21139.

21. Zhou Q, Cummings RD: **L-14 lectin recognition of laminin and its promotion of *in vitro* cell adhesion.** *Arch Biochem Biophys* 1993, **300**:6-17.

22. Sharon N: **When lectin meets oligosaccharide.** *Nat Struct Biol* 1994, **1**:843-845.

23. Bourne Y, Bolgiano B, Liao DI, Strecker G, Cantau P, Herzberg O, Feizi T, Cambillau C: **Crosslinking of mammalian lectin (galectin-1) by complex biantennary saccharides.** *Nat Struct Biol* 1994, **1**:863-870.

24. Di Virgilio S, Glushka J, Moremen K, Pierce M: **Enzymatic synthesis of natural and 13C enriched linear poly-*N*-acetyllactosamines as ligands for galectin-1.** *Glycobiology* 1999, **9**:353-364.

25. Pace KE, Lee C, Stewart PL, Baum LG: **Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1.** *J Immunol* 1999, **163**:3801-3811.

26. Liu FT: **Galectins: a new family of regulators of inflammation.** *Clin Immunol* 2000, **97**:79-88.

27. Perillo NL, Uittenbogaart C, Nguyen J, Baum LG: **Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes.** *J Exp Med* 1997, **185**:1851-1858.

28. Vespa GNR, Lewis LA, Kozak KR, Moran M, Nguyen JT, Baum LG, Miceli MC: **Galectin-1 specifically modulates TCR signals to enhance TCR apoptosis, but inhibit IL-2 production and proliferation.** *J Immunol* 1999, **162**:799-806.

29. Symons A, Cooper DN, Barclay AN: **Characterization of the interaction between galectin-1 and lymphocyte glycoproteins CD45 and Thy-1.** *Glycobiology* 2000, **10**:559-563.

30. Walzel H, Blach M, Hirabayashi J, Kasai KI, Brock J: **Involvement of CD2 and CD3 in galectin-1 induced signaling in human Jurkat T-cells.** *Glycobiology* 2000, **10**:131-140.

31. Miceli MC, Moran M, Chung CD, Patel VP, Low T, Zinnanti W: **Co-stimulation and counter-stimulation: lipid raft clustering controls TCR signaling and functional outcomes.** *Semin Immunol* 2001, **13**:115-128.

32. Chung CD, Patel VP, Moran M, Lewis LA, Carrie Miceli M: **Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction.** *J Immunol* 2000, **165**:3722-3729.

33. Rabinovich GA, Ariel A, Hershkoviz R, Hirabayashi J, Kasai KI, Lider O: **Specific inhibition of T-cell adhesion to extracellular matrix and proinflammatory cytokine secretion by human recombinant galectin-1.** *Immunology* 1999, **97**:100-106.

34. • Furtak V, Hatcher F, Ochieng J: **Galectin-3 mediates the endocytosis of β-1 integrins by breast carcinoma cells.** *Biochem Biophys Res Commun* 2001, **289**:845-850.
Previous work proposed that galectin antagonism of integrin-mediated cell binding to the ECM was due to competition between galectins and integrins for matrix glycoproteins. This work provides evidence for a novel mechanism by which galectin-3 antagonizes integrins by removing them from the cell surface. This study also adds to the growing body of work showing that galectin-3 has both intracellular and extracellular functions.

35. Hikita C, Vijayakumar S, Takito J, Erdjument-Bromage H, Tempst P, Al-Awqati Q: **Induction of terminal differentiation in epithelial cells requires polymerization of hensin by galectin 3.** *J Cell Biol* 2000, **151**:1235-1246.

36. Levy Y, Arbel-Goren R, Hadari YR, Eshhar S, Ronen D, Elhanany E, Geiger B, Zick Y: **Galectin-8 functions as a matricellular modulator of cell adhesion.** *J Biol Chem* 2001, **276**:31285-31295.

37. Van Meer G: **The different hues of lipid rafts.** *Science* 2002, **296**:855-857.

38. Kopitz J, von Reitzenstein C, Burchert M, Cantz M, Gabius HJ: **Galectin-1 is a major receptor for ganglioside GM1, a product of the growth-controlling activity of a cell surface ganglioside sialidase, on human neuroblastoma cells in culture.** *J Biol Chem* 1998, **273**:11205-11211.

39. Leitinger B, Hogg N: **The involvement of lipid rafts in the regulation of integrin function.** *J Cell Sci* 2002, **115**:963-972.

40. Mitchell JS, Kanca O, McIntyre BW: **Lipid microdomain clustering induces a redistribution of antigen recognition and adhesion molecules on human T lymphocytes.** *J Immunol* 2002, **168**:2737-2744.

41. Hansen GH, Immerdal L, Thorsen E, Niels-Christiansen LL, Nystrom BT, Demant EJ, Danielsen EM: **Lipid rafts exist as stable cholesterol-independent microdomains in the brush border membrane of enterocytes.** *J Biol Chem* 2001, **276**:32338-32344.

42. Cho M, Cummings RD: **Galectin-1, a β-galactoside-binding lectin in Chinese hamster ovary cells. II. Localization and biosynthesis.** *J Biol Chem* 1995, **270**:5207-5212.

43. Cooper DN, Barondes SH: **Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory mechanism.** *J Cell Biol* 1990, **110**:1681-1691.

44. •• Moody AM, Chui D, Reche PA, Priatel JJ, Marth JD, Reinherz EL: **Developmentally regulated glycosylation of the CD8αβ coreceptor stalk modulates ligand binding.** *Cell* 2001, **107**:501-512.
See annotation to [45••].

45. •• Daniels MA, Devine L, Miller JD, Moser JM, Lukacher AE, Altman JD, Kavathas P, Hogquist KA, Jameson SC: **CD8 binding to MHC class I molecules is influenced by T cell maturation and glycosylation.** *Immunity* 2001, **15**:1051-1061.

These two papers [44••,45••] describe a novel role for glycosylation in regulating the interactions of antigen-presenting molecules during T cell development in the thymus. Although the binding of MHC class I molecules to their ligand CD8 on mature peripheral T cells is highly restricted, promiscuous binding of different MHC class I molecules to immature thymocytes was observed. This promiscuous binding is inhibited by the sialylation of glycoproteins on mature thymocytes and peripheral T cells, as removal of sialic acid restored binding. Moody *et al.* [44••] attribute this, at least in part, to regulated expression of the ST3Gal I sialyltransferase late in thymocyte development. Together, these two studies [44••,45••] show that glycosylation affects the organization and presentation of cell surface receptors to regulate ligand binding.

46. Baum LG: **Developing a taste for sweets.** *Immunity* 2002, **16**:5-8.

47. Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS: **T cell receptor signaling precedes immunological synapse formation.** *Science* 2002, **295**:1539-1542.

48. Schwartz J-CD, Zhang X, Nathenson SG, Almo SC: **Structural mechanisms of costimulation.** *Nat Immunol* 2002, **3**:427-434.

49. Sato M, Nishi N, Shoji H, Seki M, Hashidate T, Hirabayashi J, Kasai Ki K, Hata Y, Suzuki S, Hirashima M, Nakamura T: **Functional analysis of the carbohydrate recognition domains and a linker peptide of galectin-9 as to eosinophil chemoattractant activity.** *Glycobiology* 2002, **12**:191-197.

50. Vyas AA, Patel HV, Fromholt SE, Heffer-Lauc M, Vyas KA, Dang J, Schachner M, Schnaar RL: **Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (MAG), an inhibitor of nerve regeneration.** *Proc Natl Acad Sci USA* 2002, **99**:8412-8417.